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on

Molecular Mapping for Improved Salinity Tolerance in Rice

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Table of content

	Page No
Acknowledgment	i
Table of content	ii
Section 1: Information on project personnel	v
Section 2 : Executive summary	viii
Section 3:	1
1. Introduction	1
2. **Objectives of the project**	2
3. Materials and methods	2
3.1. Experimental site	2
3.2. Selection of parents	2
3.3. Hybridization	3
3.4. Molecular assessment of hybridity	3
3.4.1. Extraction of genomic DNA	3
3.4.2. Assessment of hybridity by SSR markers	3
3.5. Development of mapping population	4
3.6. Planting materials for QTL analyses	4
3.7 Establishment of hydroponic system with saline stress	4
3.8. Phenotypic assessment of the mapping population for salinity tolerance	5
3.8.1 Assessment of RILs by salinity survival Index (SSI)	6
3.8.2 Assessment of RILs by modified Standard Evaluation Score (SES) of visual salt injury.	6
3.8.3. Assessment of RILs by growth parameters	7
3.8.4. Assessment of RILs by shoot Na^+ and K^+ concentrations	7
3.9. Genotypic assessment of mapping population	7

3.9.1. DNA extraction 7

3.9.2. Selection of SSR markers 7

3.9.3. Screening of parents for polymorphism 13

3.9.4. Genotyping of RILs 14

3.10. Analysis of variance 14

3.11. Linkage map construction 14

3.12 QTL analysis 14

4. Results 15

4.1. Molecular assessment of hybridity.

4.2. Development of mapping population 15

4.3. Variation of phenotypic traits in mapping population under salinity stress 17

4.4. Frequency distributions of the RIL population 17

4.5. Correlations among phenotypic characters for saline stress. 18

4.6. Polymorphism between parental genotypes with molecular markers in chromosome 1. 25

4.7. Genotyping of RILs with polymorphic markers in chromosome 1 25

4.8. Construction of a linkage map in chromosome 1 25

4.9. Detection of QTLs in chromosome 1 35

4.9.1. Detection of QTLs by single marker analysis method. 35

4.9.2. Mapping of QTLs by single marker regression. 35

4.9.3. Mapping of QTLs by composite interval mapping. 36

4.10. Polymorphism between parental genotypes with molecular markers in chromosome 3 44

4.11. Genotyping of RILs with polymorphic markers in chromosome 3. 44

4.12. Construction of linkage map and identification of QTLs in chromosome 3 44

4.13. Polymorphism between parental genotypes and genotyping with polymorphic markers in chromosome 4. 51

4.14. Construction of linkage map of chromosome 4. 51

4.15. Detection of QTLs in chromosome 4 59

4.15.1. Detection of QTLs by single marker analysis method 59

4.15.2. Mapping of QTLs by single marker regression 59

4.15.3 Mapping of QTLs by composite interval mapping 60

4.16. Polymorphism in parents with molecular markers in chromosome 12 and genotyping 66

4.17. Analysis of extremely tolerant and susceptible RILs for other chromosome 69

4.18. Genotyping of extreme RILs for polymorphic markers in other chromosomes 69

4.19. Identification of RILs acquiring favorable alleles 74

5. Discussion 76

5.1. Hybridization and Development of mapping population 76

5.2. Variation of phenotypic traits in mapping population under salinity stress 77

5.3. Mapping of QTLs for salt tolerance 78

6. Conclusion And Recommendation 81

7. References 83

8. Problems If Any, Encountered during the Course of the Project 88

9. Major Findings 88

Section 4 89

Section 5 92

Section 6 92

Section 7 93

Section 1:

Information on the project/project Personnel

i) Grant No: RG/2011/BT/02

ii) Title of the Project: Molecular Mapping for Improved Salinity Tolerance in Rice

iii) Principle Investigator: (Prof/Dr./Mr./Ms.)

Prof. N. S. Kottearachchi, Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka.

iv) Co-Investigators:

Co-Investigator 1:

Dr. W. L. G. Samarasinghe, Research Officer, Biotechnology Unit, Rice Research and Development Institute, Department of Agriculture, Batalegoda, Ibbagamuwa.

Collaborator 1:

Mrs. D. R. Gimhani, Lecturer, Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka.

Collaborator – 2

Mr. D.N. Sirisena, Research Officer, Soil Science Unit. Rice Research and Development Institute Department of Agriculture, Batalegoda, Ibbagamuwa

v) Institute where Research was carried out:

Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka and Rice Research Development Institute (RRDI), Batalegoda, Ibbagamuwa

vi) Date of award: 18.06.2012

vii) Date of completion of the project: 18.06.2015

viii) Total allocation of funds (Rs): Rs1,786,000.00

ix) Total spent (Rs): Rs 1,782,949.58

x) Number of Research Students employed: 2 (One research student, Ms Buddhika Dahanayake, completed MPhil degree with the support of NSF- research student allowance and MPhil registration fee. Another student, Ms DR Gimhani was able to complete her PhD with the support of consumables and mapping population provided by this research grant)

xi) Postgraduate degree completed with dates:

- Ms Buddhika Dahanayake : Defended the MPhil thesis on 25th August 2015 (Annexure 1and 2)
- Ms DR Gimhani : Submitted the draft copies of PhD thesis on 25th August 2015 for the evaluation (Annexure 3)

xii) Number of Technical Assistants and/or laborers employed and period of service: Nill

xiii) Publications/Communications arising from the project during the reporting period

Peer reviewed journals

1. Dahanayaka, B.A., Gimhani, D.R., Kottearachchi, N.S. and W. L. G. Samarasinghe. (2015). Assessment of salinity tolerance and analysis of SSR markers linked with *Saltol* QTL in Sri Lankan rice genotypes. **American Journal of Experimental Agriculture 9(5), 1-10.** (Annexure 4)

2. Wijerathna, Y. M. A. M., Kottearachchi, N. S., Gimhani, D. R. and Sirisena, D. N. (2014) . Exploration of relationship between fragrant gene and growth performances of fragrant rice (*Oryza sativa* L.) seedlings under salinity stress. **Journal of Experimental Biology and Agricultural Sciences 2(1), 7-12.** (Annexure 5)

3. **Gimhani, D.R.,**. Kottearachchi, N.S. and Samarasinghe, W.L.G. **(2014).** Microsatellite Marker Based Hybridity Assessment for Salinity Tolerance in Rice- Journal of Agricultural Sciences- Sabaragamuwa University of Sri Lanka –9(2), 96-100. (Annexure 6)

Abstracts

1. Gimhani, D.R.,. Kottearachchi, N.S., Samarasinghe, W.L.G. and Gregorio, G.B.**(2014).** Mapping of Salinity Tolerant QTLs on Chromosome 1 in Rice (*Oryza sativa*) using RIls Derived from At354 and Bg352. **4th International Rice Congress, 27 October-1 November 2014, Bangkok, Thailand.** (Annexure 7)

2. Dahanayaka, B.A., Kottearachchi, N.S., Gimhani, D.R., Samarasighe, W.L.G. **(2014).** Analysis of QTL for salinity tolerance on chromosome 4 in rice. **4th International Rice Congress, 27 October-1 November 2014, Bangkok, Thailand.** (Annexure 8)

3. Kottearachchi, N.S., Dahanayaka, B.A. and Gimhani,. D.R. **(2014).** Assessment of seedling tolerance of Sri Lankan rice (*Oryza sativa*) germplasm under salt stress using SSR markers linked with *Saltol* QTL. **4th International Rice Congress, 27 October-1 November 2014, Bangkok, Thailand.** (Annexure 9)

4. Dahanayaka, B.A., Kottearachchi, N.S., Gimhani, D.R. and Samarasighe, W.LG. Allelic diversity and seedling tolerance of some rice (*Oryza sativa*) germplasms under salt stress. **1st Ruhuna International Science and Technology Conference, University of Ruhuna, Matara, January 22-23, (2014).** (Annexure 10)

5. Dahanayaka, B.A., Kottearachchi, N.S., Gimhani, D.R. and Samarasighe, W.LG. Progress towards mapping of a QTL for salinity tolerance on chromosome 4 in rice- **2014-1st Wayamba University International symposium, 29-30 August 2014.** (Annexure 11)

6. Gimhani, D.R., Kottearachchi, N.S. and Samarasinghe, W.L.G. **(2013).** Development of Mapping Population for Salinity Tolerance using At354 and Bg352 Rice Varieties- **7th International Rice Genetics symposium, 5 to 8 November 2013,Manila, Philippines** (Annexure 12)

7. Gimhani, D.R., Kotrearachchi, N.S. and Samarasinghe, W.L.G.- **2012-** Microsatellite Marker Based Hybridity Assessment; an Approach Towards Development of Mapping Population for Salinity Tolerance in Rice- **Proceedings of the Young Scientist Forum Symposium-18th** January 2013**, (organized by National Science Technology Commission-NASTEC)** (Annexure 13)

8. Gimhani, D.R., Kotrearachchi, N.S. and Samarasinghe, W.L.G.- **2012** - Mapping population development for salinity tolerant QTLs in rice and microsatellite marker based hybridity assessment- **Indo - Sri Lankan International Conference on "Agrobiotechnology for Sustainable Development" (ABSD-12)** Organized by University of Ruhuna, University of Peradeniya, University of Colombo in Sri Lanka in collaboration with the Department of Microbiology Dr. Babasaheb Ambedkar Marathwada University, Aurangabad, Maharashtra, India (Annexure 14)

Manuscript in preparation

Presently 2 manuscripts are being written under following titles.

1. SNP marker based Mapping of QTLs for Salt tolerance in rice
2. Mapping of QTLs in chromosome 1 and 4 for salt tolerance in rice

Section 2:

Executive Summary of the Project

A cross between At354, a salt tolerant parent and Bg352, a salt susceptible parent was made to produce recombinant inbred line (RIL) population aiming at identifying salinity tolerant QTLs. Hybridity of F_1 progeny was confirmed using microsatellite markers and 6 true hybrids were advanced to produce 281 lines of F_5 generation by single seed method.

For the investigation of salinity tolerant QTLs, nine phenotypic parameters related to salinity tolerance were assessed in randomly selected 100 RILs of F5 population under hydroponics supplemented with 100 mM NaCl concentration (12 dS/m). For the genotyping of the RILs 158 SSR markers were surveyed and of them 45 markers that were polymorphic between At354 and Bg352 were used for genotype-trait association analysis. Linkage maps were constructed for chromosome 1, 3 and 4 as it was a prerequisite for linkage based QTL mapping. QTLs were identified by single marker analysis, simple interval mapping and composite interval mapping. Composite Interval mapping revealed 6 QTLs distributed in chromosome 1 and chromosome 4 namely, *qSSI1,qSL1,qSNK1,qSL4,qSNK4* and *qSSI,* explaining 8.9% to 16% of the phenotypic variations of SSI, shoot length and shoot Na^+/K^+ ratio. In both instances of *qSSI4* and *qSL4* LOD peak laid between RM280 and RM3843 which has about 3Mbp difference, thereby suggesting to be used as potential flanking markers for breeding studies of salt tolerance. The RILs accumulated with 5 promising QTLs were identified for further analysis on agronomic traits and salinity tolerance in reproductive stage as an application of project output.

Section 3

1. INTRODUCTION

Rice (Oryza sativa L.) is one of the most important grain in Asian countries and it is consumed by two third of the world population as their staple food. With the escalating growth rate of world population the demand for the rice is increasing year by year. Therefore, usage of marginal lands like saline affected areas is important to withstand the challenges in rice production. It is estimated that within 30 years world population will increase by 2 more billions (Mohanty et al., 2012) hence the food production has to be increased accordingly. As rice is the food for more than half of the population food security aspects should be mainly focused on rice production. As the lands accessible for agricultural purpose are also decreasing it is necessary to maximize the utility of available lands.

Salinity is considered as a serious constrain in rice growing areas and among abiotic stress, salinity has been recognized as the second most wide spread problem for reduction in growth and productivity of rice (Gregorio et al., 1997b) in all over the world. Millions of hectares in coastal regions face salinity due to marine/brackish water intrusion to the ground while the inland salinity can be occurred due to many reasons like poor irrigation, digestion of ores, human activities etc. Salinity has covered over 7% of land worldwide (Szabolcs, 1994) which can be technically used for the crop cultivation. In Sri Lanka approximately 13% of the irrigated lands are affected by salinity stress (Thiruchelvam. and Pathmarajah, 1999) and this percentage is gradually increasing in both coastal regions and inlands (Sirisena and Herath, 2009).

Most of the rice varieties are extremely sensitive to salinity during young seedling stage and early development stage (Heenan et al., 1988). Mainly there are two methods by which rice seedlings are affected by excess salt. Effect of excessive amount of salt present in the soil can rapidly damage the plant by disturbing the osmotic balance to result sudden death of leaves. Prolong damages can be occurred due to the accumulation of salts in tissues which can be interfered with the metabolism like protein synthesis of the rice seedling. Transport of excess salts to older tissues, acquired salt responsive stomata and synthesis of osmoprotectants are some of the mechanisms that are involved with salinity tolerance of rice.

According to the past research studies, QTLs associated with different mechanisms of salinity tolerance located in various chromosomes were identified under different genetic background of rice (Koyama et al., 2001 Singh et al., 2007; Haq et al., 2010; Singh and Flowers, 2010). Of them a major QTL (*Saltol*), was identified on short arm of chromosome 1 using RILs derived from IR29/Pokkali cross accounting 43% of the variation in shoot Na^+/K^+ ratio at the seedling stage (Gregorio, 1997a ; Bonilla et al., 2002). Therefore identification of these QTLs and pyramid them all into one line would enable to develop superior cultivars which can be used in marginal land that are abandoned due to salinity. Considering the broad objective of developing salinity tolerant cultivars by compiling many salinity tolerant QTLs, this project was planned aiming at identifying salinity tolerant QTLs using a mapping population derived from native rice cross and this report present the project implementation procedure, results and outcomes of the project.

2. OBJECTIVES OF THE PROJECT

General Objective:

To identify and combine QTLs/genes controlling different physiological mechanisms that lead to achieve higher level of salt tolerance in popular rice varieties to be used in salt-affected areas in Sri Lanka.

Specific Objectives: (as stated in the original proposal)

1. To discover salinity tolerant QTLs, *Saltol* 1 and other Pokkali inherited QTLs using SSR technique in elite rice background

2. To discover novel salinity tolerant QTLs, appeared in At354 x Bg352 cross

3. To identify promising flanking markers for efficiently transfer of the QTLs into popular varieties

4. To develop Recombinant Inbred Line population to be utilized in future molecular mapping

5. To identify Recombinant Inbred Lines that has accumulated promising QTLs to introduce as intermediate parents for future breeding programs

6. Human capacity building in the area of plant molecular breeding

3.0. MATERIALS AND METHOD

3.1. Experimental site

The study was mainly conducted at the Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, Makandura Gonawila. Hybridization work was carried out, in collaboration with the Rice Research and Development Institute (RRDI), Batalagoda. Some analysis were conducted in the Genetech, Sri Lanka and the International Rice Research institute, Philippines where due reference were mentioned in the method.

3.2. Selection of parents

At354 (*O. sativa) indica* rice variety derived from Pokkali and Bg94-1 was selected as salinity tolerant parent as it exhibits extreme tolerance to salinity in Sri Lanka. Breeders have used Pokkali landrace as a donor of the salinity tolerance in At354 rice variety. Bg352 (*O. sativa) indica* rice variety derived from Bg380/Bg367-4 cross was selected as a salinity susceptible cultivar. Both of them are recommended varieties with 3 ½ months maturity period, by the Department of Agriculture, Sri Lanka as they contain elite characters. Usually both At354 and Bg352 are capable of giving comparatively higher yield and the highest yield recorded in both varieties is around 5-6 t/ha. Eventhough Bg352 is susceptible to salinity, it is characterized as a variety with resistance to brown plant hopper and blast and presently this is the most popular variety in farmers in Pollonnaruwa district (Department of Agriculture, 2013). Other than the salinity

tolerance associated with At354, it is also resistant to lodging. Therefore, the population derived from the cross of At354 and Bg352 likely to be gained salinity tolerant traits along with the other desirable improved traits presented in both cultivars.

3.3. Hybridization

Since the selected parents, At354 and Bg352 are in the same maturity class of 3 ½ months with the same flowering time; both varieties were established in the field at the same time period. Then hybridization of At354 was done with Bg352 which was used as the pollen parent.

3.4. Molecular assessment of hybridity

3.4.1. Extraction of genomic DNA

Genomic DNA was extracted from well grown putative F_1 seedlings and two parents according to the method described in http://rgp.dna.affrc.go.jp/ rgp/protocols/QTL.pdf.

The leaf pieces were homogenized with 300 µl of DNA extraction buffer (1 M KCl, 1 M Tris-HCl pH 8.0, 0.5 M EDTA pH 8.0). Homogenized mixtures were incubated at 70 °C for 20 min. Extracts were centrifuged at 13,000 rpm for 15 min under room temperature. Then, 100 µl of ice cold iso-propanol was added into new eppendorf tubes, and the supernatant of the above centrifuged samples were transferred into them. Solutions were mixed gently to facilitate the precipitation of DNA. After mixing, tubes were kept at 4 °C for 15-30 min and centrifuged at 13,000 rpm for 15 min under room temperature. DNA pellets were washed with 150 µl of 70% ice cold ethanol by centrifuging at 13,000 rpm for 10 min. Supernatants were removed and pellets were air dried and dissolved in 200 µl of 1/10 TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0).

3.4.2. Assessment of hybridity by SSR markers

According to the Thomson, et al., (2010) two SSR markers *viz.*, RM1287 and RM493 which were closely associated with the region of *Saltol* QTL in chromosome 1 were selected for the genetically screening of parents for polymorphism (Table 3.1). The primer sequence information was obtained from the published sequence database of Gramene (http://www.gramene.org).

Genomic DNA of 14 well grown F_1 seedlings was amplified along with the parents using 2 microsatellite markers with the 15 µl PCR mixture consisted of 5 µl of genomic DNA, 1.5 µl of 10X PCR buffer, 1.2 µl of dNTPs (2.5 mM of each dNTP), 0.8 µl of primer mixture (20 µM of each Forward and Reverse primer) (Integrated DNA Technologies, USA) and 0.25 µl of *Taq* DNA polymerase (5U/ µl, Sigma, USA). Final volume of the mixture was adjusted to 15 µl by adding sterile distilled water. PCR amplification was performed using thermal cycler, BIORAD (USA) and amplification profile consisted of initial denaturing at 95 °C for 5 minutes followed by 35 cycles of 1 minute at 95 °C, 30 seconds at relevant annealing temperature (Table 3.1), 1 minute at 72 °C and final extension cycle of 5 minutes at 72 °C. About 7.5 µl of amplified PCR products mixed with 2µl of 6X loading dye were subjected to electrophoresis in 3% agarose gel containing ethidium bromide (0.5 µg / ml), in 0.5X TBE buffer at 5V/cm.

After electrophoresis the gel was visualized under UV light using Quntum ST4 (Vilber Lourmat, France) gel documentation system and hybridity of F_1 individuals was confirmed by comparing complementary alleles of parents.

Table 3.1. Sequences, and annealing temperatures of selected SSR markers used for hybridity assessment

Locus name	Primer sequence	SSR motif	Annealing Temp. (°C)
RM493	F- TAGCTCCAACAGGATCGACC R-GTACGTAAACGCGGAAGGTG	$(CTT)_n$	56
RM1287	F-GTGAAGAAAGCATGGTAAATG R-CTCAGCTTGCTTGTGGTTAG	$(AG)_n$	53

F- Forward primer
R- Reverse primer

3.5. Development of mapping population

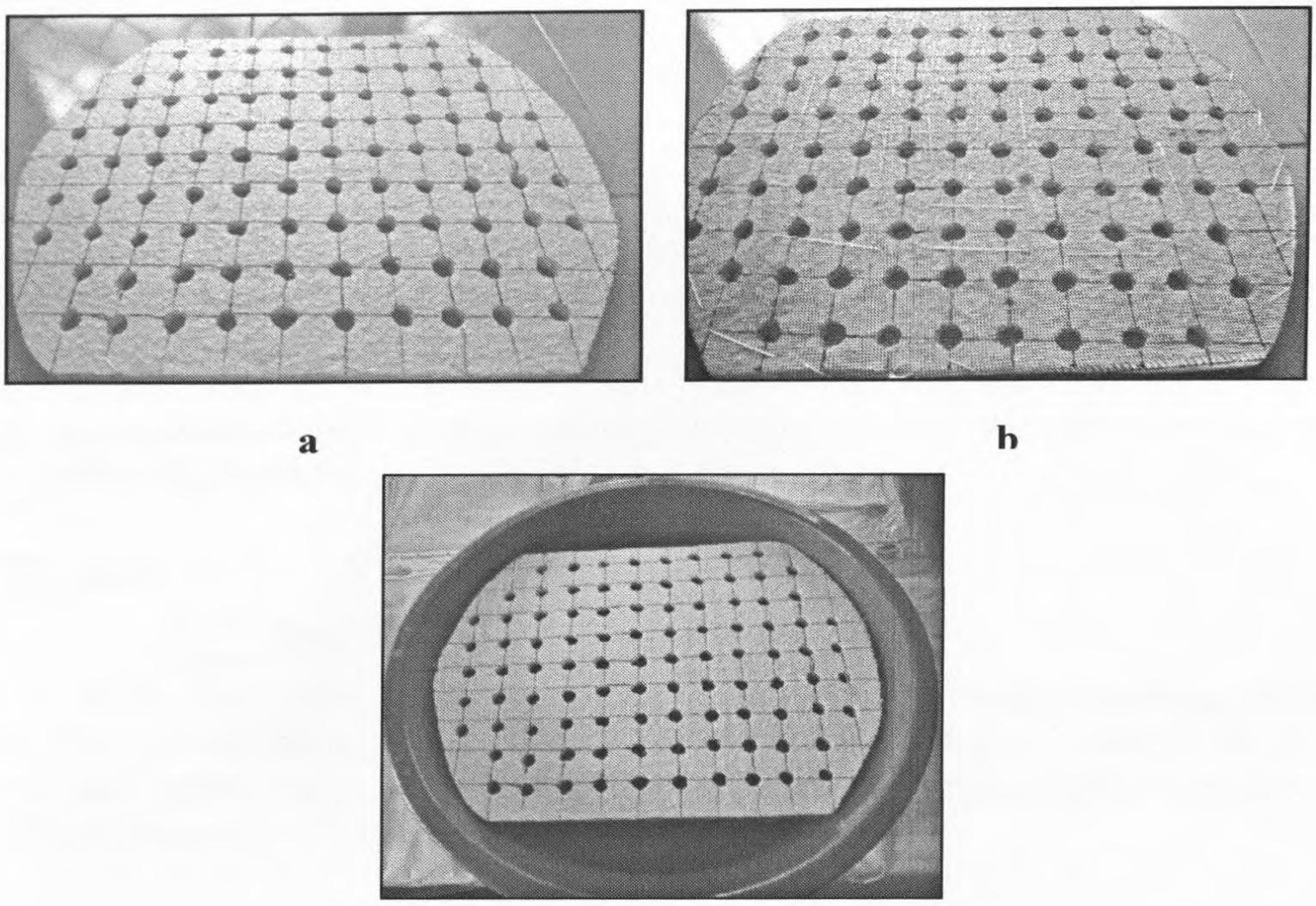
True F_1 hybrids were selected from 14 well grown F_1 seedlings based on SSR marker banding pattern that showed heterozygosity and they were transplanted in the field. F_1 generation was advanced to F_2 population by self pollination of F_1 individuals. Seeds of F_2 segregants were planted in the nursery bed at RRDI for 2 weeks and then F_2 seedlings were transplanted in the field. F_3 generation was raised through self pollination of F_2 segregants. Thereafter, F_3 generation was advanced to subsequent generations through inbreeding towards F_5 generation by single seed descent method (SSD) to produce recombinant inbred line population (RILs) (Figure 4.2).

3.6. Planting materials for QTL analyses

Out of 281 RILs derived from At354 and Bg352 cross, 100 RILs (F5) were randomly selected for mapping of QTLs for salt tolerance.

3.7 Establishment of hydroponic system with saline stress

The mapping population consists of 100 RILs and two parents (At354 and Bg352) were assessed for seedling stage salinity tolerance using a hydroponics system, according to the screening technique developed by Gregorio et al., (1997b). Screening for salinity tolerance was conducted under the natural daylight in the plant house at WUSL. The Experimental set up was designed according to the Randomized Complete Block Design. Two blocks were used with 10 individual plants from each line per block along with the control. Screening set up was prepared by placing the Styrofoam seedling floats with nylon net bottom consists of 100 holes in the basin with 23 L capacity (Figure 3.1).



a **b**

c

Figure 3.1. Hydropoinic system established for screening at seedling stage salinity tolerance.
a-Styrofoam seedling float
b- Nylon net bottom
c- 23L basin filled Yoshida nutrient solution

Seeds were placed on moistened filter papers in petri dishes and incubated at the room temperature for 48 h for germination. After 2 days once seedlings have developed primary roots about 2-3 cm long, they were transplanted to the seedling floats suspended on basin filled with tap water. After 3 days when seedlings were well established, floats were transferred to the basins containing 22 L of Yoshida nutrient solution (Yoshida et al., 1976). Initially nutrient solution in each basin was salinized to EC of 6 dS m^{-1} by adding appropriate amount of NaCl (Analytical grade) except for control. After 2 days salinity was increased upto EC of 12 dS m^{-1} (100 mM) by adding appropriate amount of NaCl. The pH of the solution was maintained at 5.0 on a daily basis, by adding either 1M NaOH or HCl. Nutrient solution was renewed in every 8 days. Screening was conducted for 21 days from the date of salinization to EC of 12 dS m^{-1}. Simultaneously control setup was also maintained under the similar experimental condition without salinization.

3.8. Phenotypic assessment of the mapping population for salinity tolerance

The mapping population was phenotypically assessed for salinity tolerance using 9 morpho-physiological indices *viz*., standard evaluation score (SES), salinity survival index (SSI), shoot length (SL), root length (RL), shoot dry weight (SDW), root dry

weight (RDW), shoot Na^+ concentration (SNC), shoot K^+ concentration (SKC) and shoot Na^+/K^+ ratio (SNK) under hydroponics supplemented with 12 dS/m.

3.8.1 Assessment of RILs by salinity survival Index (SSI)

A quantitative parameter called salinity survival index (SSI) was used to assess the survival potential of RILs under salt stress which ranges from 0-1. SSI was measured by giving maximum weight for the plants that survived throughout the whole period (SSI =1.000) while minimum weight was given for the plants that died at earliest possible (closer to 0). Accordingly, the number of dead plants was counted on every three days after salinization (upto 12 dS m^{-1}) and the respective seedling survival percentage of each day was calculated until 21 days after salinization (DAS). The SSI was calculated using the following formula,

$$SSI = \sum_{k=1}^{n} D_k S_k \Big/ \left(\sum_{k=1}^{n} D_k\right) 100$$

Where, D is the Day after salinization (DAS), S is the survival percentage of that particular day, n is the total period in DAS (in this experiment n is 21 DAS), D_k is the DAS at k^{th} data collection, S_k is the survival percentage of k^{th} data collection and k=1, 2, 3….n. (Wijerathna et al., 2014).

3.8.2 Assessment of RILs by modified Standard Evaluation Score (SES) of visual salt injury.

RILs were also evaluated for salt tolerance based on the modified Standard Evaluation Score (SES) (1-9 scale are described in Table 3.2) of visual salt injury at 14 days and 21 days after salinization, as reported by International Rice Research Institute (Gregorio et al., 1997b).

Table 3.2. Modified Standard Evaluation Score (SES) of visual salt injury at seedling stage

SES	Description	Tolerance
1	Normal growth, Only old leaves show white tips while no symptoms on young leaves	Highly tolerant
3	Near normal growth, but only leaf tips burn, few old leaves become whitish partially	Tolerant
5	Growth severely retarded; most old leaves severely injured, few young leaves elongating	Moderately tolerant
7	Complete cessation of growth; most old leaves dried; only few young leaves still green	Susceptible
9	Almost all plants dead or drying	Highly susceptible

3.8.3. Assessment of RILs by growth parameters

At the 21st day of salinity treatment, shoot and root samples of each line were harvested and respective shoot length (SL) and root length (RL) of the seedlings were measured. Then shoot and root materials of each line were oven dried for 3 days at 70 °C and relevant shoot and root dry weight were also measured.

3.8.4. Assessment of RILs by shoot Na^+ and K^+ concentrations

For the determination of shoot Na^+ and K^+ concentrations, hydroponic system was established separately as mentioned in the section 3.7 and whole shoot was harvested from each line (3 plants from each line) 21 days after salinization,. Shoots were washed 3 times using distilled water and then dried at 70 °C for 3 days. After drying, samples were ground to a fine powder and 10 mg of the powder was used for the extraction in 10 ml of 0.1 N acetic acid incubated in a water bath of 90°C. Incubation was continued for 2 h at least till the sample was completely digested. Once extracted solution was cooled upto room temperature, solution was filtered using Whatman 1 filter paper and diluted 10 times. Shoot Na^+ and K^+ concentrations were measured by flame emission spectrometry using a flame photometer at 589 nm.

3.9. Genotypic assessment of mapping population

3.9.1. DNA extraction

Genomic DNA was extracted from randomly selected 100 RILs that was used to assess phenotypic parameters according to the method presented in http://rgp.dna.affrc.go.jp/rgp/protocols/QTL.pdf. Detailed method was described in above paragraph 3.4.1.

3.9.2. Selection of SSR markers

At first experiments were targeted to detect previously reported QTL, *Saltol*, inherited from Pokkali, in chromosome 1 as At354 contained the Pokkali genetic background. Therefore, 57 SSR markers located in the close proximity to *Saltol* region was selected based on Thomson, et al., (2010), McCouch et al., (1997), Mohammadi-Nejad et al., (2008) and Haq et al., (2008) (Table 3.3). Of them 36 SSR markers were associated with the main *Saltol* QTL region and 22 markers were located outside the *Saltol* QTL region. The primer sequence information was obtained from the published sequence database (www.gramene.org). Then, in order to find out novel QTLs, ninety two SSR markers and 9 InDel markers (Lu et al., 2009), collectively 101 DNA primers (Table 3.4) covering rest of the 11 chromosomes were used. The sequences were obtained from previously reported articles (Thomson et al., 2010; McCouch et al., 2002) and data available at Gramene database (www.gramene.org).

Table 3.3. Sequences and annealing temperature of the selected 57 SSR markers located on chromosome 1.

	Primer Name	Annealing T. (°C)	Primer Sequence 5'-3'
1	RM8094*	56	AAGTTTGTACACATCGTATACA CGCGACCAGTACTACTACTA
2	RM10764*	56	AGATGTCGCCTGATCTTGCATCG GATCGACCAGGTTGCATTAACAGC
3	RM10772*	55	GCACACCATGCAAATCAATGC CAGAAACCTCATCTCCACCTTCC
4	RM10745*	58	TGACGAATTGACACACCGAGTACG ACTTCACCGTCGGCAACATGG
5	RM14	56	CCGAGGAGAGGAGTTCGAC GTGCCAATTTCCTCGAAAAA
6	RM3412*	55	AAAGCAGGTTTTCCTCCTCC CCCATGTGCAATGTGTCTTC
7	RM7075*	55	TATGGACTGGAGCAAACCTC GGCACAGCACCAATGTCTC
8	RM140*	58	TGCCTCTTCCCTGGCTCCCCTG GGCATGCCGAATGAAATGCATG
9	RM243	58	GATCTGCAGACTGCAGTTGC AGCTGCAACGATGTTGTCC
10	RM10852*	58	GAATTTCTAGGCCATGAGAGC AACGGAGGGAGTATATGTTAGCC
11	RM9*	58	GGTGCCATTGTCGTCCTC ACGGCCCTCATCACCTTC
12	RM10710*	52	GCTTCGATCGATGAGAAAGTAGAGG GAATCTCCCATCCTTCCCTTCC
13	RM10782*	52	CTACTGATATGCCCGTACCAACG CCAAACTGGAGTAGTATTAGGTGTGG
14	RM10746*	52	ATGACTCTTCGCCATTCCATAGC ACCATGGTCAGCCATCACTAGG
15	RM151	52	GGCTGCTCATCAGCTGCATGCG TCGGCAGTGGTAGAGTTTGATCTGC
16	RM1344	52	CTGCAATCCGAGTAGGAAGC TGAGCATTCACTCCGATCTG
17	RM10718*	52	TAACTCGCCACCGATGATTTCG CGACCAACGTGCAATAGTACAAGC
18	RM10776*	52	CCACGGTCAACAGAGAGATTCC TTTCACCGACGAGGCTATCG
19	RM581	52	ACATGCGTGATCAACAATCG AATTGGATGTGGATGCACG
20	RM562*	53	CACAACCCACAAACAGCAAG CTTCCCCCAAAGTTTTAGCC
21	RM493*	56	TAGCTCCAACAGGATCGACC GTACGTAAACGCGGAAGGTG
22	RM8115*	50	TATATAGTAAATTTGTTTGGTGTAGG ACAGATGGATATTATAAGAAGTAACA
23	RM6711*	56	TAGTGATAGGGGTGGTGTGG TTACAAGCATGGGAGTTGGG
24	RM1287*	53	GTGAAGAAAGCATGGTAAATG CTCAGCTTGCTTGTGGTTAG
25	RM3825	58	AAAGCCCCCAAAAGCAGTAC GTGAAACTCTGGGGTGTTCG
26	RM583	55	AGATCCATCCCTGTGGAGAG GCGAACTCGCGTTGTAATC
27	RM10754*	55	GCAGTAGCAGCGGTAGGAGTAGC CCTGCTGCCTTGAATTGAACC
28	RM1020	58	AACTTTCCCACCACCACCGCGG AGCAGCAGCAAGCCAGCAAGCG

29	RM129	55	TCTCTCCGGAGCCAAGGCGAGG CGAGCCACGACGCGATGTACCC
30	RM10968 *	55	TCCTTCACCTCCGCCTCAAACC CCATGGAGACTAGGTGGAACTTGAGG
31	RM1329	55	GAGCTCAATCGAATCTAGACC ATTGACATTCCTTTGCTTTG
32	RM10825*	60	GGACACAAGTCCATGATCCTATCC GTTTCCTTTCCATCCTTGTTGC
33	RM10864*	60	GAGGTGAGTGAGACTTGACAGTGC GCTCATCATCCAACCACAGTCC
34	RM11757*	60	GCTTGTTGCCTGTGAACAGTAGC TGTCAGCATGCAACATCAATCC
35	RM10694*	60	TTTCCCTGGTTTCAAGCTTACG AGTACGGTACCTTGATGGTAGAAAGG
36	RM7643	55	TCCTCCTATTCGGTCGAAAC ACCAACGAAATACCGGCAC
37	RM11008	60	TTTGGATGGTCATTAGCCTCTGG ATCAACCTTGCATGCTGTCTTCC
38	RM11300	60	GGTGAGGGAGGTACCGAACTAGG AACTAGGGCGCTGGGAGAGG
39	RM472	60	CCATGGCCTGAGAGAGAGAG AGCTAAATGGCCATACGGTG
40	RM10793*	60	GACTTGCCAACTCCTTCAATTCG TCGTCGAGTAGCTTCCCTCTCTACC
41	RM11874	55	CCACTAGCAGATGATCACAGACG GAGCACCTCATAAGGGTTTCAGC
42	RM486	55	CCCCCCTCTCTCTCTCTCTC AGCCACATCAACAGCTTGC
43	AP3206*	55	TTCTCATCGCACCATCTCTG GGAGGAGGAGAGGAAGAAG
44	RM3412b*	55	TCATGATGGATCTCTGAGGTG GGGAGGATGCACTAATCTTTC
45	RM10720*	55	GCAAACGTCTACGTGAGAAACAAGC GCATGTGGTGCCTTAACATTTGG
46	RM10748*	55	CATCGGTGACCACCTTCTCC CATCGGTGACCACCTTCTCC
47	RM10843*	55	CACCTCTTCTGCCTCCTATCATGC GTTTCTTCGCGAAATCGTGTGG
48	RM10800*	55	CGTACGCCCTCACATCACCTTTCC CTCTCCGGGAGCTCACTTGTCG
49	RM10871*	55	GAGGCTGTAACGTAGACGATGAACC AAGCCTGCTAGAGAGGCCCAACC
50	RM10890*	55	GCTTCGGCTCTTCATTCACTGG GCGATTATAGGAGCGCTATGTGG
51	RM10287	55	GTATTCCTTGCTGCTGCTGATGG GACTGGAGATGTGATCGGAAACC
52	RM10927*	55	TGGATCCCACTAATCCAAATGC GAAAGACTCCTTCCAATGTTAGGC
53	RM3252	55	GGTAACTTTGTTCCCATGCC GGTCAATCATGCATGCAAGC
54	RM10115	55	ACAAGACGAGGTAACACGCAAGC GCGAAGGATCAACGATGATATGG
55	RM10701*	55	GAGACACGGCACAATATACAACG TTCTATCTCCGACCTCTTCTCAAGG
56	RM10711*	55	GCTTCGATCGATGAGAAAGTAGAGG GAATCTCCCATCCTTCCCTTCC
57	RM10829*	55	CATCCGTGGAGCAAGGAGAGG CCTAGCTAATTGGAGTCCGGGTTGG

*SSR markers located within the *Saltol* region (10.8 Mb - 16.4 Mb)

Table 3.4 Sequences and annealing temperature of the selected 101 SSR/InDel markers located on chromosome 2-12.

	Primer	Annealing Tem.(^{0}C)	Chromosome	Sequence from 5'-3'
1	RM148	55	3	ATACAACATTAGGGATGAGGCTGG TCCTTAAAGGTGGTGCAATGCGAG
2	RM3867	55	3	TTGACTGGAACATCGAGCTC ATCCCCTCTACACCGTACCC
3	RM6329	55	3	CCCTGGATGAAAAGCACAAG GAAGTTGTAGATGCCCCATC
4	RM251	55	3	GAATGGCAATGGCGCTAG ATGCGGTTCAAGATTCGATC
5	RM293	55	3	TCGTTGGGAGGTATGGTACC CTTTATCTGATCCTTGGGAAGG
6	RM218	55	3	TGGTCAAACCAAGGTCCTTC GACATACATTCTACCCCCGG
7	RM60	57	3	AGTCCCATGTTCCACTTCCG ATGGCTACTGCCTGTACTAC
8	RM22	57	3	GGTTTGGGAGCCCATAATCT CTGGGCTTCTTTCACTCGTC
9	RM545	57	3	CAATGGCAGAGACCCAAAAG CTGGCATGTAACGACAGTGG
10	RM7	55	3	TTCGCCATGAAGTCTCTCG CCTCCCATCATTTCGTTGTT
11	RM282	57	3	CTGTGTCGAAAGGCTGCAC CAGTCCTGTGTTGCAGCAAG
12	RM16	57	3	CGCTAGGGCAGCATCTAAA AACACAGCAGGTACGCGC
13	RM570	57	3	GTTCTTCAACTCCCAGTGCG TGACGATGTGGAAGAGCAAG
14	RM231	55	3	CCAGATTATTTCCTGAGGTC CACTTGCATAGTTCTGCATTG
15	RM5626	57	3	ATCAGTCGGTCATAAACGCC ACCTTCCTCTTCTGCTGCTG
16	RM554	57	3	GTTCGTCCGTCTCTCGTCTC CCCAAAAATCTGTGCCTCTC
17	RM563	57	3	CGACCCTAGGGTTTCTCC CTCGACGTCGTGGAAAGC
18	RM3864	57	3	AGTCAACCTTGGGGGTAAGG AGATACTGCCCGTGTCATCC
19	RM130	59	3	TGTTGCTTGCCCTCACGCGAAG GGTCGCGTGCTTGGTTTGGTTC
20	RM6283	57	3	TGGAGACTGAGCTGATGCC TCAGGTGGTCGGTTCCTTAC
21	RM411	57	3	ACACCAACTCTTGCCTGCAT TGAAGCAAAAACATGGCTAGG
22	RM36	57	3	CAACTATGCACCATTGTCGC GTACTCCACAAGACCGTACC
23	R4M17*	52	4	AGTGCTCGGTTTTGTTTTC GTCAGATATAATTGATGGATGTA
24	R4M43*	52	4	CTTGAACCTGAGTGAGTGG CGATGAAAATGATGTCTA
25	R4M50*	52	4	TTTTGTGAAACTTGACCCTC GCGTCCATGTCTTTATTGTG
26	R4M13*	52	4	TACACGGTAGACATCCAACA ATGATTTAACCGTAGATTGG
27	RM127	60	4	GTGGGATAGCTGCGTCGCGTCG

				AGGCCAGGGTGTTGGCATGCTG
28	RM273	55	4	GAAGCCGTCGTGAAGTTACC GTTTCCTACCTGATCGCGAC
29	RM518	58	4	CTCTTCACTCACTCACCATGG ATCCATCTGGAGCAAGCAAC
30	RM307	57	4	GTACTACCGACCTACCGTTCAC CTGCTATGCATGAACTGCTC
31	RM3843	57	4	ACCCTACTCCCAACAGTCCC GGGGTCGTACGCTCATGTC
32	RM241	55	4	GAGCCAAATAAGATCGCTGA TGCAAGCAGCAGATTTAGTG
33	RM5749	57	4	GTGACCACATCTATATCGCTCG ATGGCAAGGTTGGATCAGTC
34	RM317	59	4	CATACTTACCAGTTCACCGCC CTGGAGAGTGTCAGCTAGTTGA
35	RM2636	52	4	CGGAGGAAGTACCTTATAAA CTTCTCAGATTCTTGTGTGT
36	RM335	57	4	GTACACACCCACATCGAGAAG GCTCTATGCGAGTATCCATGG
37	RM261	57	4	CTACTTCTCCCCTTGTGTCG TGTACCATCGCCAAATCTCC
38	RM470	57	4	TCCTCATCGGCTTCTTCTTC AGAACCCGTTCTACGTCACG
39	RM280	55	4	ACACGATCCACTTTGCGC TGTGTCTTGAGCAGCCAGG
40	RM142	59	4	CTCGCTATCGCCATCGCCATCG TCGAGCCATCGCTGGATGGAGG
41	RM119	61	4	TAGTGCCGATCGATGTAACG CATATGGTTTTGACAAAGCG
42	RM559	59	4	TTCCCCTCCTTTTATGGTGC TGTTCTCCTCAGTCACTGCG
43	RM1272	57	4	GGCCGTTGGTTCTAAAATC TGCGCAGTATCATCGGCGAG
44	RM6303	57	4	CTTCGATCCGGTGACGAC AACGAAAGCGAAGCTGTCTC
45	RM17708	60	4	CATCCCCCTGCTGCTGCTGCTG CGCCGGATGTGTGGGACTAGCG
46	RM17693	59	4	TCTTCCACAACATGCTCTTCACC GCTGCTATGTCAACTAGTGTCTCACG
47	RM1113	57	4	GGGCGCATGTGTATTTCTTC TGGGGAAAAACCACAAGCC
48	RM3335	51	4	TAATCCACTGTGTCATTTAA ACCATCATCTTGTACCTAGT
49	R12M27*	55	12	ATTTCATTGCCATCAGTT GTAATCTTCTATCCGTTCA
50	RM102	60	12	AACTTTCCCACCACCACCGCGG AGCAGCAGCAAGCCAGCAAGCG
51	RM28102	60	12	CACTAATTCTTCGGCTCCACTTTAGG GTGGAAGCTCCGAGAAAGTGC
52	RM1261	57	12	GTCCATGCCCAAGACACAAC GTTACATCATGGGTGACCCC
53	RM519	57	12	AGAGAGCCCCTAAATTTCCG AGGTACGCTCACCTGTGGAC
54	RM247	55	12	TAGTGCCGATCGATGTAACG CATATGGTTTTGACAAGCG
55	RM463	57	12	TTCCCCTCCTTTTATGGTGC TGTTCTCCTCAGTCACTGCG
56	RM270	55	12	GGCCGTTGGTTCTAAAATC TGCGCAGTATCATCGGCGAG
57	RM511	57	12	CTTCGATCCGGTGACGAC

				AACGAAAGCGAAGCTGTCTC
58	Ba76H14	55	12	GAAACGGGGTCAAATAAGC GGGTTCGTCCAACAGGAGTA
59	RM277	63	12	CGGTCAAATCATCACCTGAC CAAGGCTTGCAAGGGAAG
60	RM3326	56	12	CTCATCACCATCGTCACCAC TCGTCGGGAGAGAGAGAGAG
61	RM491	56	12	ACATGATGCGTAGCGAGTTG CTCTCCCTTCCCAATTCCTC
62	RM13197	62	2	AAACCCTCCGGCTCATTCTTGC ACTCGAATCGTATCGGCTTGAGG
63	RM300	57	2	GCTTAAGGACTTCTGCGAACC CAACAGCGATCCACATCATC
64	RM6318	57	2	TGCTGCTTCTGTCCAGTGAG GGATCATAACAAGTGCCTCG
65	RM5404	57	2	GGCCATCCATCTCCTGTATG GACACACACAGGGTTGGTTG
66	RM154	65	2	ACCCTCTCCGCCTCGCCTCCTC CTCCTCCTCCTGCGACCGCTCC
67	R5M30*	55	5	CTCAATTTCACCCATCCC CGCTCCGTCTCCAACCTC
68	RM 527	55	6	GGCTCGATCTAGAAAATCCG TTGCACAGGTTGCGATAGAG
69	R6M14*	52	6	AAATGTCCATGTGTTTGCTTC CATGTGTGGAATGTGGTTG
70	RM20224	58	6	AGTATGAAAGTCGGTGACGATGG GAGATGTCACGTCTTCACTTAGGG
71	RM248	55	7	TCCTTGTGAAATCTGGTCCC GTAGCCTAGCATGGTGCATG
72	RM7076	55	7	TGGTTCGATTCGGATTTC AAGCTATTCACAAGCAGCTC
73	RM11	58	7	TCTCCTCTTCCCCCGATC ATAGCGGGCGAGGCTTAG
74	RM234	55	7	ACAGTATCCAAGGCCCTGG CACGTGAGACAAAGACGGAG
75	RM20848	60	7	AAAGTGGGCACTGAGATACAACG AGCGAAGGCAGTGAAGTTTCG
76	RM21539	60	7	GCCCAACTACTTCGACAGCTTCC CAATGACCTGAGTAGCATCCAAGG
77	RM214	55	7	CTGATGATAGAAACCTCTTCTC AAGAACAGCTGACTTCACAA
78	RM3635	57	7	CGTGAGAGCGTGAGAGACAG ACTTTGGTGTTCCCTCCCTC
79	RM10	57	7	TTGTCAAGAGGAGGCATCG CAGAATGGGAAATGGGTCC
80	RM5436	57	7	GCATCCCGGTGACTAGTACG TGTGCATGTGGTAAGGAACC
81	RM5353	55	8	ACCCTCGATCTCCTAGGCTG TCTACTCCAAACCCATTGCC
82	RM149	60	8	GCTGACCAACGAACCTAGGCCG GTTGGAAGCCTTTCCTCGTAACACG
83	RM25	51	8	GGAAAGAATGATCTTTTCATGG CTACCATCAAAACCAATGTTC
84	RM242	55	9	GGCCAACGTGTGTATGTCTC TATATGCCAAGACGGATGGG
85	RM7175	58	9	ACAGTAAACGTGGTGCCTCC AGAAGTAGCCTCGAGGACCC
86	RM296	58	9	CACATGGCACCAACCTCC GCCAAGTCATTCACTACTCTG
87	RM201	55	9	CTCGTTTATTACCTACAGTACC

				CTACCTCCTTTCTAGACCGATA
88	RM3164	55	9	TCCTCCTGCTAGCTGCCTAG TCGCCTTCCTTTTCACTCAC
89	RM24330	60	9	AAT CCG CGG GAG CAA TCA ACC CGA TGA CCA ATG ACG AGG TGA GG
90	R10M30*	52	10	CCCTAAAAATAGAGCAACCT ACCCATAATACTACCAATCAAC
91	RM304	52	10	TCAAACCGGCACATATAAGAC GATAGGGAGCTGAAGGAGATG
92	RM228	58	10	CTGGCCATTAGTCCTTGG GCTTGCGGCTCTGCTTAC
93	RM258	58	10	TGCTGTATGTAGCTCGCACC TGGCCTTTAAAGCTGTCGC
94	RM24855	58	10	TTGGTGTTGTCCACCTCCACATACC CGTCGATACCAATGGCGACTACC
95	RM24909	58	10	AGCGAATAGGCCCTTAATTTCC GACCCTGTTTAGTTCACGAGACG
96	R11M23*	52	11	AAGGTTGACAAGGACAGAAG TCGCAGGAATGGATAAAA
97	RM229	58	11	CACTCACACGAACGACTGAC CGCAGGTTCTTGTGAAATGT
98	RM21	55	11	ACAGTATTCCGTAGGCACGG GCTCCATGAGGGTGGTAGAG
99	RM209	55	11	ATATGAGTTGCTGTCGTGCG CAACTTGCATCCTCCCCTCC
100	RM224	55	11	CACTCACACGAACGACTGAC CGCAGGTTCTTGTGAAATGT
101	RM332	55	11	GCGAAGGCGAAGGTGAAG CATGAGTGATCTCACTCACCC

* *InDel markers*

3.9.3. Screening of parents for polymorphism

At354 and Bg352 parents were screened for the polymorphism with 158 SSR/InDel markers using polymerase chain reaction (PCR) which was conducted as mentioned in the paragraph 3.4.2.

Amplified PCR products were first analyzed using 3% agarose containing ethidium bromide (0.5 µg / ml), in 0.5X TBE buffer at 5V/cm. After electrophoresis the gel was visualized under UV light using Quntum ST4 (Vilber Lourmat, France) gel documentation system. Based on the discrimination of parental alleles at the respective SSR locus, polymorphic SSR markers between parents were selected. Some PCR products, of which respective parental alleles could not be resolved to detect polymorphism using 3% agarose gel, were subjected to 8% polyacralmide gel electrophoresis (PAGE) as it has higher resolution power than the agarose gel. Accordingly, amplified DNA fragments were run on 8% polyacrylamide gel using Biometra S_2 Model (German) which is available in Genetech Pvt LTd, Sri Lanka followed by 0.1 % silver staining. Few primers were subjected to 8% PAGE using Dual Triple-Wide Mini-Vertical System (C.B.S. Scientific, CA, USA) which is avaialble in International Rice Research Institute, Philippine with 1X TBE buffer (Tris- Borate EDTA) at 100 V using SYBR-Safe (Invitrogen, Carlsbad, USA) staining (5 µl of SYBR-Safe per 50 ml of nanopure water). The gel was visualized using Alpha Innotech gel documentation system.

3.9.4. Genotyping of RILs

The markers that were found polymorphic between parents were used to genotype RILs under the same PCR conditions mentioned in 3.4.2. Amplified PCR products of the RIL DNA were analyzed with the same conditions used for the parents. Using the gel profiles, manual scoring was conducted to each allele amplified by respective SSR markers. Based on the parental alleles, allelic band similar to At354 parental allele was designated as "A" genotype. Allelic band similar to Bg352 parental allele was designated as "B" genotype. Individuals with heterozygous genotype where both parental alleles were present at the respective SSR locus were designated as "h".

3.10. Analysis of variance

Significant differences among the mapping population for the parameters such as shoot length, root length, root dry weight, shoot dry weight, SSI, shoot Na^+/K^+ ratio, shoot Na^+ concentration and shoot K^+ concentration were detected by Analysis of variance (ANOVA) using SPSS 16.0 (SPSS, 2007). All the measured phenotypic indices of parents were compared with Student's t-test. Correlation coefficients also were calculated and frequency distributions were drawn using MINITAB V17.0 (Minitab17, 2010).

3.11. Linkage map construction

Linkage analysis of SSR markers was performed using JoinMap4.1 software (Stam, 1993). Linkage groups were created at a logarithm of odds ratio (LOD) score threshold of 2. Marker orders were estimated using the maximum likelihood mapping algorithm and recombination fractions were converted to map distances (cM) via the Kosambi mapping function (Kosambi, 1944).

3.12 QTL analysis

Before proceeding to the detailed QTL analysis, initially each marker genotype was analyzed by "single marker analysis method" using student's t-test to detect whether there is an association between marker locus and the quantitative trait. In this method each respective trait measured in the mapping population was partitioned according to parental marker genotypes resulted in the mapping population.

Each marker locus was also analyzed by single marker regression (SMR) method which is similar to student's t-test using Qgene 4.3.10 software. Detailed QTL analysis was performed by employing composite interval mapping (CIM) using Qgene 4.3.10 (Joehanes and Nelson, 2008) in order to detect the putative QTLs more precisely.

In present study for QTL mapping, physical position (Mb) of the SSR markers based on the Nipponbare genome was used for an approximate estimation of marker distances in centi Morgan (cM) by multiplying the physical position in Mb by a factor of 4. For this estimation of genetic distances between markers, one million bases on a rice chromosome were assumed to be equivalent to approximately 3.92 cM (IRGSP, 2005; Thomson et al., 2010; Sandu et al., 2014; Ye et al., 2011). These cM positions were used for QTL mapping by SMR and CIM using Qgene 4.3.10.

CIM was performed with the standard model with a walk speed of 2 cM and setting forward cofactor selection as auto. Permutation test (1000 times) was performed for each trait with SMR and CIM approaches to establish an experiment-wise LOD threshold value at the 0.05 and 0.01 significance level (Doerge and Churchill, 1996; Churchill and Doerge, 1994). The proportion of the observed phenotypic variance explained by each QTL was estimated by the coefficient of determination (R^2) (McCouch et al., 1997). QTL names were designated following the standard rice QTL nomenclature (McCouch and CGSNL, 2008).

4. RESULTS

4.1. Molecular assessment of hybridity.

In present study, At354 (donor parent for salinity tolerance) was crossed with Bg352 (salinity susceptible recurrent parent) using At354 as a female partner. According to the results of the hybridization, F_1 progeny consisting of 119 F_1 seeds was developed and of them only 14 F_1 seeds successfully produced vigorous F_1 seedlings. Subsequently, all 14 F_1 seedlings were subjected to molecular assessment of the hybridity in order to select the true hybrids. Accordingly, RM1287 and RM493 markers exhibited polymorphism between At354 and Bg352 alleles at the respective loci. These two markers were used to examine the hybridity of 14 well grown F_1 individuals. Consequently, hybridity of 6 F_1 individuals was confirmed proving their heterozygosity at the respective loci representing two specific alleles of both parents (Figure 4.1). Rest of the 8 F_1 individuals were confirmed as off-types as they exhibited only one of the alleles of parents (Figure 4.1).

4.2. Development of mapping population

Selected 6 true hybrids were established in the field and of them, 350 F_2 segregants were raised successfully followed by advancing of subsequent generations upto F_5 generation by single seed descent method (Figure 4.2). The final F_5 progeny consisted of 281 lines of recombinant inbred lines which could be utilized as the mapping population for salt tolerance.

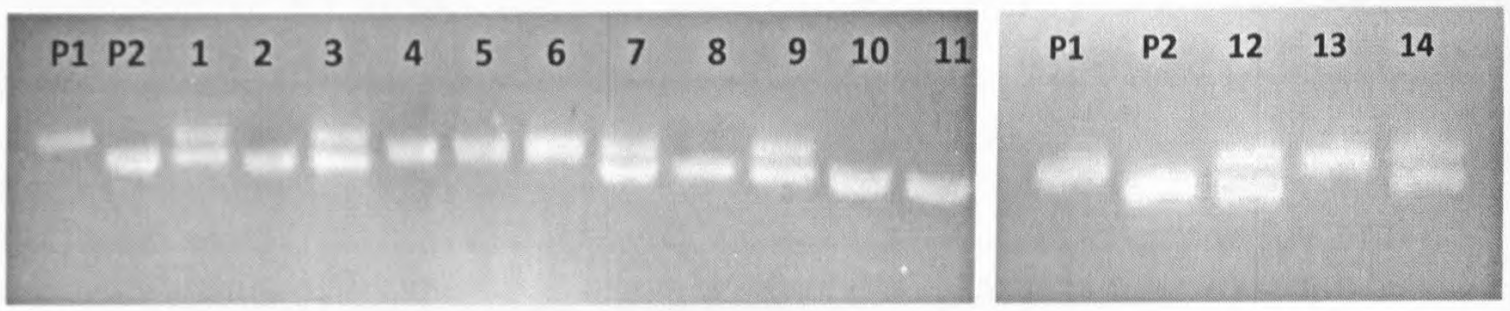


Figure 4.1. PCR profiles generated with At354, Bg352 and F_1 individuals using RM1287 and RM493 SSR markers (a) PCR profile generated by RM1287 (b) PCR profile generated by RM493 SSR markers.
Lane P1- At354 parent, Lane P2-Bg352 parent, Lane 1-14- F_1 individuals

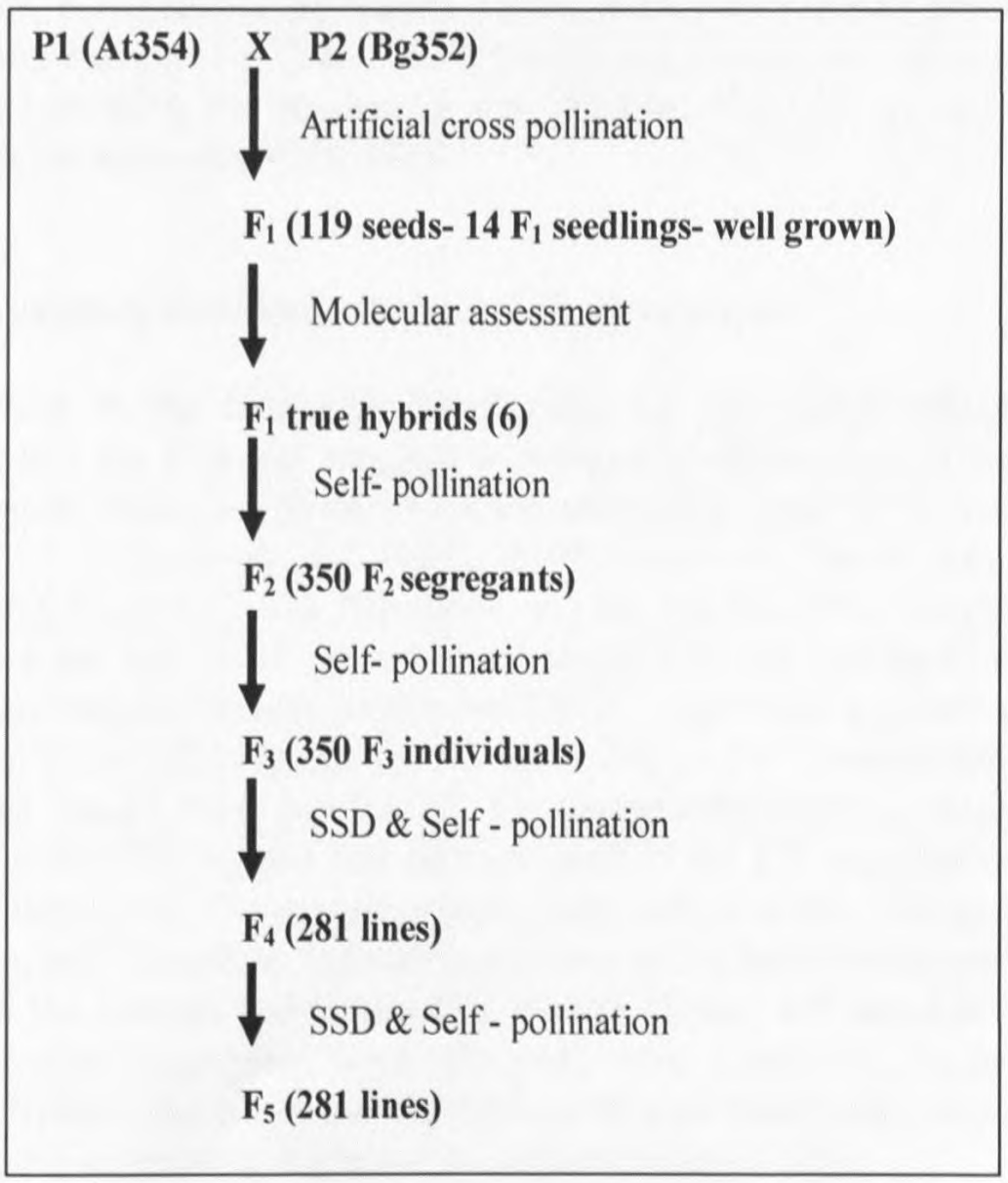


Figure 4.2. Schematic representation showing the number of lines produced by each advanced generation.

4.3. Variation of phenotypic traits in mapping population under salinity stress

In this preliminary study subset of RIL population consists of 100 RILs were evaluated for salinity tolerance phenotypically along with 2 parents At354 and Bg352 using 9 morpho-physiological parameters *viz.*, Salinty Survival Index (SSI), modified Standard Evaluation score (SES), shoot length (SL), root length(RL), shoot dry weight (SDW), root dry weight (RDW), shoot Na^+ concentration (SNC), shoot K^+ concentration (SKC) and shoot Na^+/K^+ ratio (SNK) (Figure 4.3 and 4.4). Mean performances of the parents and the RIL population for all the measured morpho-physiological indices under saline stress are summarized in the Table 4.1. Accordingly, selected both parents were significantly different for all the measured indices indicating their divergent performances under saline stress condition (Table 4.1) (Figure 4.3). Mean values observed in RIL population were indicated in Table 4.2. In the present study, association of higher coefficient of variation (CV) with dry biomass of shoot (CV- 34.99%) and root (CV- 47.48%) showed comparatively wider variation than the variation of shoot and root length among RILs under salt stress. Mean shoot length (21.44 cm) and root length (14.86 cm) in the population were closer to the average of the tolerant and susceptible parent (20.72 cm and 14.9 cm respectively) ranging from 7.92 cm to 34.45 cm and 8.5 cm to 26.05 cm respectively (Table 4.1).

For the detection of QTLs in chromosomes, it is essential to evaluate the phenotypic variation among RIL population for the considered characters. Results of the analysis of variance, revealed that differences among RILs were highly significant ($p<0.01$) for all the traits except SES (Table 4.3). Significant difference was not observed among 2 blocks indicating the absence of the blocking effect in the experimental set-up, on phenotypic responses of the RILs.

4.4. Frequency distributions of the RIL population

According to the frequency distributions all the morpho-physiological parameters except SES (as SES was assigned as categorical basis) showed continuous and normal distribution which lay down within the acceptable range of skeweness below +1.5 and above 1.5 (Tabachnick and Fidell, 2013), exhibiting quantitative nature of the traits (Figure 4.5 and 4.6). The maximum and the minimum root lengths observed in RILs were 8.5 cm and 26.05 cm, for shoot length 7.92 cm and 34.45 cm. The variation of shoot dry weight, root dry weight and Na^+/K^+ were 0.056 g to 0.45 g, 0.016 g to 0.204 g and 0.696 to 6.722 respectively. For shoot Na^+ and K^+ concentrations maximum values obtained were 1.9360 and 0.6580, the lowest measurements were 0.3542 and 0.2132 respectively. The highest SSI demonstrated in the RIL population was 0.938 and the lowest was 0.104. Transgressive segregants were also observed across all the measured traits in both directions indicating superior and inferior performances of certain RILs beyond the tolerant and susceptible parents (Figure 4.5 and 4.6). However, very few transgressive segregants were observed with shoot Na^+ concentration, shoot K^+ concentration and shoot Na^+/K^+ ratio as two parental means existed at the lower and upper extremities with respective to each trait (Figure 4.6).

4.5. Correlations among phenotypic characters for saline stress.

According to the correlation analysis of the studied traits, salinity survival index (SSI) was significantly, strongly and positively correlated with shoot length *(r=0.719)* at 0.001 significance level (Table 4.4). Root length *(r=0.338, P < 0.001)*, shoot dry weight (*r*= 0.246, *P < 0.05*) and root dry weight (*r=0.430, P < 0.01*) were also showed significant association with SSI indicating the increment of these traits along with the increment of the SSI (Table 4.4). However, it was noted that based on the value of the correlation coefficient, strength of the association of these 3 traits with SSI was comparatively weak. And also, according to present results, it was observed that shoot Na^+ concentration was inversely correlated with SSI (*r = -0.367*), at 0.001 significance level with a comparatively weak association between these two traits. In addition, strong positive correlations were also observed between pair of traits of shoot length and root length (*r=0.607, P < 0.001*), shoot dry weight and root dry weight (*r=0.719, P < 0.001*), shoot Na^+ concentration and shoot Na^+/K^+ ratio (*r=0.849, P < 0.001*) and shoot K^+ concentration and shoot Na^+/K^+ ratio (*r=0.626, P < 0.001*) (Table 4.4).

Table 4.1. Phenotypic variations of 9 traits in two parents (At354, Bg352) and RIL population under salinity tress (EC= 12 dS/m)

Trait	Means of Parents		RIL population					
	At354	Bg352	Mean	Min	Max	SD[a]	CV[b] %	Skewn -ess
SES	3	7	7.07	4.3	9	1.366	19.32	-
SL (cm)	26.67***	14.78***	21.44	7.92	34.45	6.098	28.44	0.10
RL (cm)	16.28***	13.53***	14.86	8.500	26.05	2.989	20.12	0.69
SDW (g)	0.200***	0.056***	0.226	0.056	0.450	0.079	34.99	0.38
RDW (g)	0.075 ***	0.0375***	0.074	0.016	0.204	0.035	47.48	0.90
SSI	0.7634	0.2232	0.5405	0.1036	0.9375	0.2191	40.54	-0.21
SNC (mmol/g)	0.3202***	1.8195***	1.0725	0.3542	1.9360	0.3604	33.61	0.55
SKC (mmol/g)	0.6396***	0.2814***	0.4052	0.2132	0.6580	0.0894	22.06	0.63
SNK (mmol/g)	0.5005***	6.512***	2.816	0.696	6.722	1.237	43.93	0.82

Modified Standard Evaluation Score (SES), Salinty Survival Index (SSI), Shoot length (SL), Root length(RL), Shoot dry weight (SDW) and Root dry weight (RDW), Shoot Na^+ concentration (SNC), Shoot K^+ concentration (SKC) and Shoot Na^+/K^+ (SNK) ratio

[a]SD-Standard deviation
[b]CV- coefficient of variation

**** significant at P<0.001 according to Student's t-test;*

Table 4.2. Mean values of RIL population for the phenotypic characters measured under salt stress.

Line No	Root length (cm)	Shoot length (cm)	Shoot dry weig-ht (g)	Root dry weig-ht (g)	SSI	Shoot Na^+ concentrat-ion (mmol/g)	Shoot K^+ concentrat-ion (mmol/g)	Shoot Na^+/K^+ ratio	SES
1	8.5	12.21	0.231	0.069	0.236	1.902	0.348	5.462	9
2	10.97	12.61	0.056	0.016	0.104	1.936	0.372	5.199	9
3	9.02	12.91	0.239	0.058	0.227	1.812	0.452	4.009	8.9
4	19	19.7	0.206	0.089	0.411	1.679	0.514	3.263	8.7
5	13.63	12.45	0.258	0.044	0.198	1.232	0.634	1.944	9
7	11.58	10.93	0.095	0.030	0.111	1.829	0.489	3.741	9
8	12.76	13.51	0.099	0.022	0.168	1.271	0.628	2.023	9
9	10.87	12.56	0.146	0.042	0.182	1.645	0.520	3.162	9
12	13.94	14.17	0.094	0.024	0.170	1.499	0.424	3.539	9
14	13.26	17.11	0.128	0.033	0.314	1.599	0.348	4.591	9
15	17.82	22.95	0.205	0.067	0.200	1.133	0.431	2.630	8.6
16	13.74	17.81	0.175	0.038	0.289	1.737	0.364	4.774	8.8
17	14.83	15.71	0.111	0.029	0.377	1.543	0.375	4.112	9
18	22.41	33.43	0.237	0.063	0.529	1.334	0.426	3.129	7.2
19	26.05	33.2	0.244	0.051	0.186	1.116	0.463	2.408	8.4
20	12.78	12.18	0.200	0.043	0.166	0.738	0.505	1.461	9
21	10.84	12.62	0.249	0.036	0.132	0.631	0.497	1.268	9
22	16.38	19.02	0.159	0.051	0.398	0.762	0.658	1.157	7.8
23	16.05	15.11	0.202	0.044	0.263	0.607	0.649	0.933	8.7
24	12.62	13.77	0.205	0.072	0.273	0.808	0.435	1.857	8.4
25	9.82	13.31	0.274	0.081	0.245	0.844	0.392	2.152	8.7
29	17.03	18.25	0.195	0.055	0.455	0.854	0.435	1.963	7.2
30	12.80	15.53	0.257	0.078	0.395	1.126	0.387	2.911	8.4
31	13.8	22.09	0.160	0.065	0.546	0.951	0.370	2.573	7
32	14.94	20.76	0.296	0.112	0.450	0.987	0.388	2.544	7.6
33	16.8	25.60	0.263	0.087	0.616	0.946	0.432	2.189	6.3
34	15.05	28.75	0.320	0.107	0.811	0.611	0.460	1.327	4.8
36	17.29	27.25	0.236	0.07	0.893	1.121	0.398	2.816	4.6
37	13.35	17.58	0.199	0.070	0.484	1.029	0.431	2.388	7.7
39	10.54	7.92	0.096	0.029	0.519	1.621	0.341	4.751	7.4
40	13.86	21.33	0.249	0.077	0.534	1.281	0.412	3.107	6.6
41	14.81	27.94	0.175	0.061	0.830	0.932	0.547	1.702	5.15
42	18.42	28.63	0.259	0.092	0.798	1.063	0.341	3.115	5.4
43	13.77	21.85	0.267	0.081	0.568	0.893	0.368	2.425	7.1
44	13.68	23.74	0.289	0.077	0.463	0.888	0.432	2.055	7.6
45	11.46	20.51	0.175	0.063	0.461	1.436	0.316	4.551	8.2
47	11.61	17.08	0.121	0.037	0.504	1.378	0.270	5.102	7.8
50	16.3	22.27	0.319	0.081	0.614	1.019	0.375	2.715	6.5
51	11.17	21.35	0.364	0.118	0.591	0.791	0.443	1.783	6.3
53	12.74	18.64	0.237	0.080	0.470	1.359	0.287	4.732	7.9
54	17.05	25.15	0.342	0.101	0.775	1.140	0.296	3.857	5.5
56	16.41	26.16	0.444	0.176	0.664	0.970	0.438	2.216	6
58	13.37	20.45	0.153	0.054	0.555	1.844	0.274	6.721	7
59	12.79	17.88	0.262	0.101	0.423	1.004	0.432	2.324	7.8
61	13.52	19.51	0.360	0.125	0.393	1.485	0.364	4.080	7.6
64	11.70	18.48	0.359	0.111	0.321	1.194	0.429	2.780	8.4
66	11.68	17.87	0.202	0.077	0.393	1.511	0.277	5.453	8
71	16.49	26.50	0.395	0.141	0.766	0.573	0.588	0.973	5.7
72	13.87	29.99	0.238	0.068	0.870	1.378	0.483	2.851	5.3

74	14.84	29.08	0.272	0.107	0.755	0.922	0.497	1.85	5.2
76	12.04	19.29	0.195	0.073	0.329	1.264	0.350	3.615	8.2
77	15.74	23.93	0.242	0.089	0.654	0.966	0.424	2.279	6
78	15.45	25.79	0.178	0.054	0.782	1.106	0.321	3.444	6
80	17.17	30.2	0.240	0.203	0.791	1.038	0.316	3.290	5.6
82	14.01	23.6	0.197	0.068	0.581	0.679	0.412	1.648	7.3
83	17.73	28.11	0.327	0.137	0.786	1.393	0.352	3.950	4.6
84	16.24	25.7	0.265	0.120	0.623	1.189	0.389	3.052	6.1
85	16.24	24.03	0.191	0.070	0.655	1.053	0.361	2.916	6.7
87	11.24	16.08	0.189	0.085	0.746	0.878	0.384	2.288	5.2
89	16.18	16.24	0.339	0.136	0.800	1.640	0.293	5.601	5.5
90	17.93	26.76	0.275	0.117	0.770	0.621	0.370	1.680	5.4
95	11.65	18.24	0.332	0.120	0.600	0.835	0.318	2.621	6.4
96	17.53	16.56	0.195	0.068	0.786	1.126	0.297	3.789	5.2
97	13.5	17.42	0.225	0.060	0.721	0.354	0.509	0.696	5.6
98	13.12	18.34	0.372	0.138	0.734	1.043	0.270	3.863	5.8
99	21.7	34.45	0.289	0.117	0.832	0.393	0.355	1.106	4.6
100	18.58	24.08	0.291	0.114	0.689	1.048	0.213	4.916	6.1
102	12.78	26.75	0.196	0.070	0.788	0.771	0.333	2.319	5.5
103	11.22	18.67	0.126	0.031	0.459	0.500	0.233	2.14	8.7
105	10.91	15.08	0.450	0.032	0.366	0.912	0.196	4.650	8.8
106 A	13.3	25.54	0.086	0.021	0.809	0.619	0.381	1.624	7.1
106	18.88	28.64	0.209	0.067	0.602	1.485	0.270	5.498	7.5
107	12.98	23.7	0.265	0.060	0.580	0.854	0.289	2.959	7.5
109	15.1	32.2	0.250	0.086	0.684	1.019	0.316	3.229	6.1
110	13.45	29.03	0.099	0.035	0.814	0.718	0.352	2.037	5.5
111	13.31	30.94	0.195	0.062	0.938	0.742	0.335	2.213	4.3
113	16.22	32.6	0.297	0.065	0.814	0.534	0.355	1.502	5.9
114	15.67	31.65	0.197	0.045	0.902	1.082	0.318	3.398	4.3
117	17.60	22.40	0.183	0.048	0.509	1.106	0.318	3.474	7.8
118	11.66	15.93	0.216	0.092	0.488	0.922	0.458	2.014	7
119	16.50	18.23	0.350	0.132	0.543	0.961	0.478	2.011	6.3
121	13.89	28.73	0.207	0.077	0.718	0.844	0.422	2.000	5.2
123	13.78	20.71	0.210	0.057	0.413	0.869	0.466	1.863	7.4
124	19.76	28.2	0.294	0.110	0.652	0.801	0.475	1.686	6.4
125	16.66	21.41	0.243	0.087	0.446	1.528	0.438	3.491	7.4
134	15.09	24.03	0.213	0.067	0.545	1.786	0.483	3.695	7.2
137	14.7	17.97	0.115	0.027	0.389	0.912	0.509	1.792	8.5
138	16.92	22.2	0.290	0.066	0.586	0.956	0.561	1.702	6.8
145	16.85	22.63	0.291	0.094	0.739	0.975	0.466	2.092	5.7
149	22.02	20.49	0.232	0.067	0.761	1.014	0.469	2.162	7.5
150	17.35	14.76	0.142	0.045	0.225	0.883	0.389	2.267	8.6
151	15.5	22.44	0.164	0.040	0.513	0.592	0.526	1.125	7.6
153	16.32	24.56	0.223	0.143	0.650	0.835	0.398	2.097	6.9
154	18.05	20.21	0.138	0.046	0.486	0.539	0.301	1.787	7.8
155	11.86	10.04	0.115	0.028	0.411	1.065	0.344	3.096	8.3
158	16.3	15.01	0.271	0.073	0.288	0.670	0.415	1.613	8.
159	16.74	22.28	0.159	0.059	0.571	0.759	0.374	2.031	7.1
162	15	24.76	0.152	0.041	0.732	0.757	0.483	1.566	5.8
163	15.71	27.72	0.148	0.110	0.909	1.029	0.426	2.412	6.7
166	18.8	30.4	0.194	0.050	0.752	0.917	0.355	2.580	6.2

Table 4.3. Analysis of variance for 8 morpho- physiological traits in the RIL population of At354 and Bg352 cross evaluated under salinity tress (EC= 12 dS/m)

	DF	MS of RL	MS of SL	MS of SDW	MS of RDW	MS of SSI	MS of SNC	MS of SKC	MS of SNK
RILs	98	108.49***	681.805***	0.0630798***	0.0100475***	0.0955***	0.2621***	0.01809***	5.946***
Blocks (Replicates)	1	3.504^{ns}	2.752^{ns}	0.003095^{ns}	0.0002985^{ns}		1.3579^{ns}	0.185442^{ns}	1.750^{ns}

Root length (RL), Shoot length (SL), Shoot dry weight (SDW), Root dry weight (RDW), Salinity Survival Index (SSI) Shoot Na^+/K^+ (SNK) ratio, Shoot Na^+ concentration (SNC) and Shoot K^+ concentration (SKC)

Degrees of freedom (DF), Means square (MS)

****significant at P<0.001* ^{ns} *not significant.*

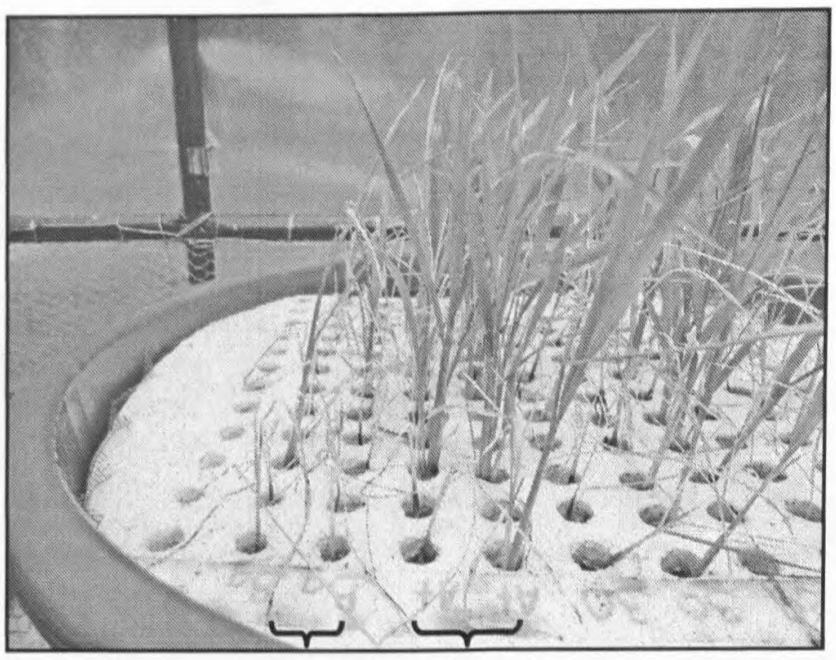


Figure 4.3. Screening of At354 and Bg352 parents for seedling stage salinity tolerance under salinity stress condition; 21 days after salinization

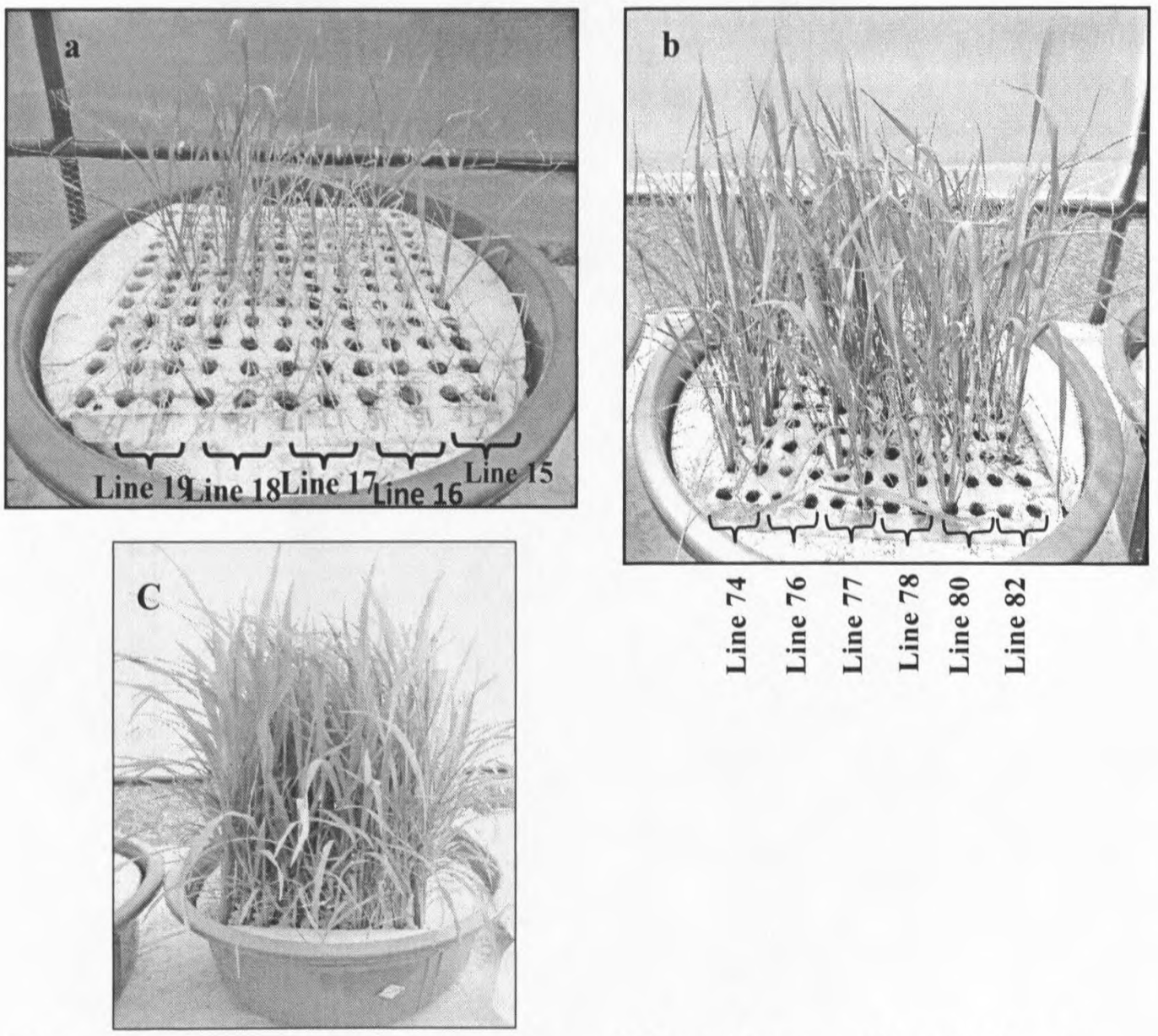
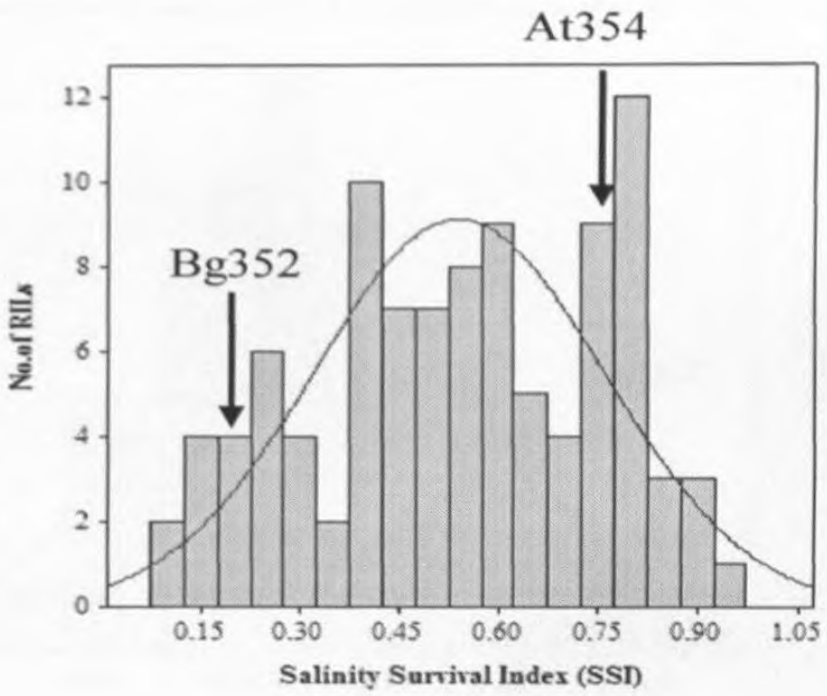
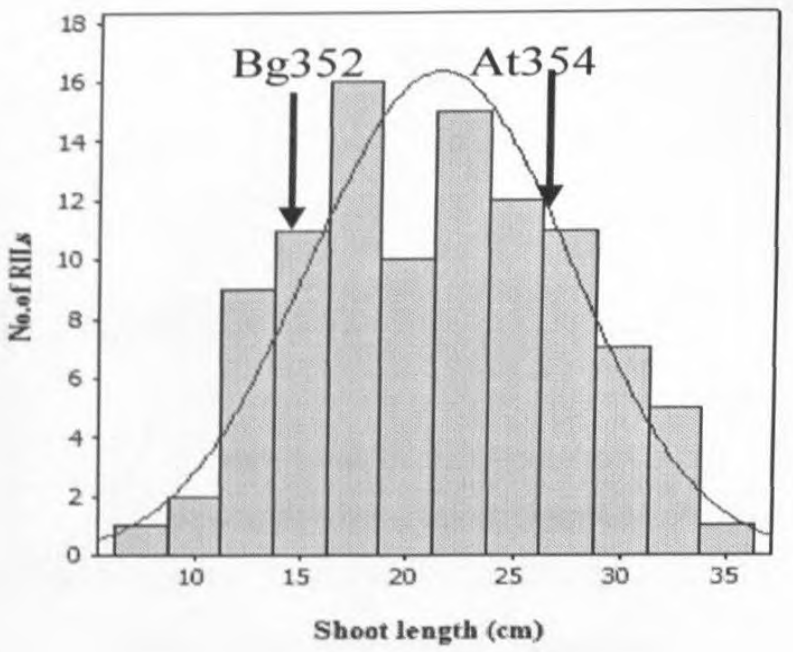
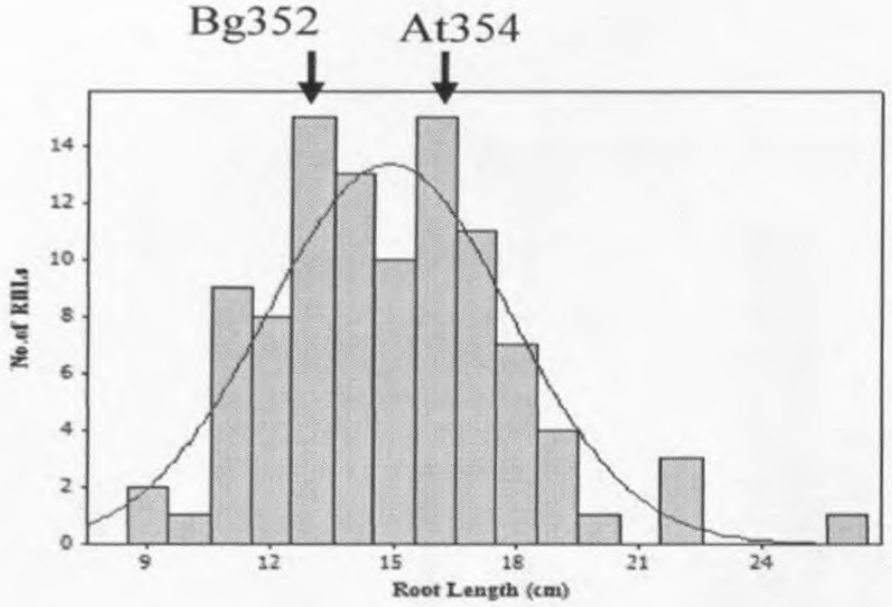
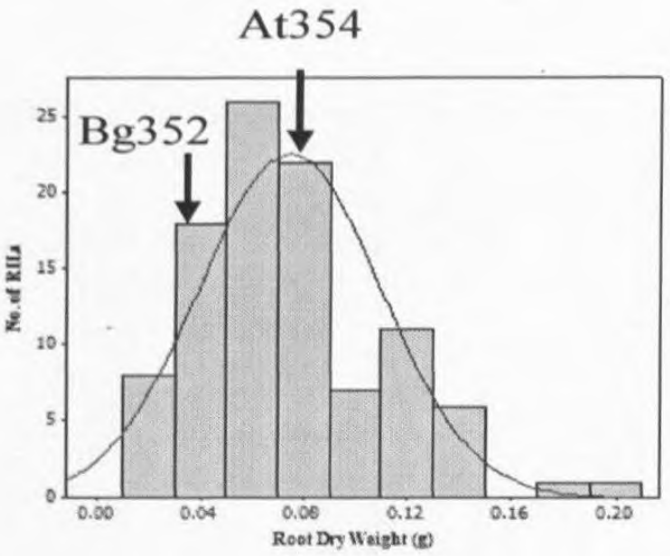


Figure 4.4. Screening RILs for seedling stage salinity tolerance under salinity stress condition (EC 12 dS/m). a and b – RIL Line No. 15-19, 74, 76,77,78,89 and 82, 21 days after salinization. c- Control set-up without salinization









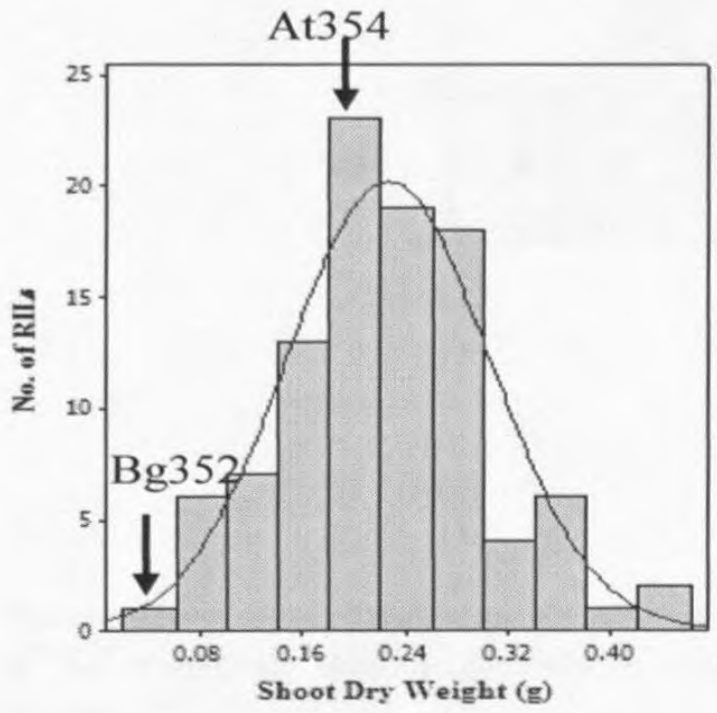
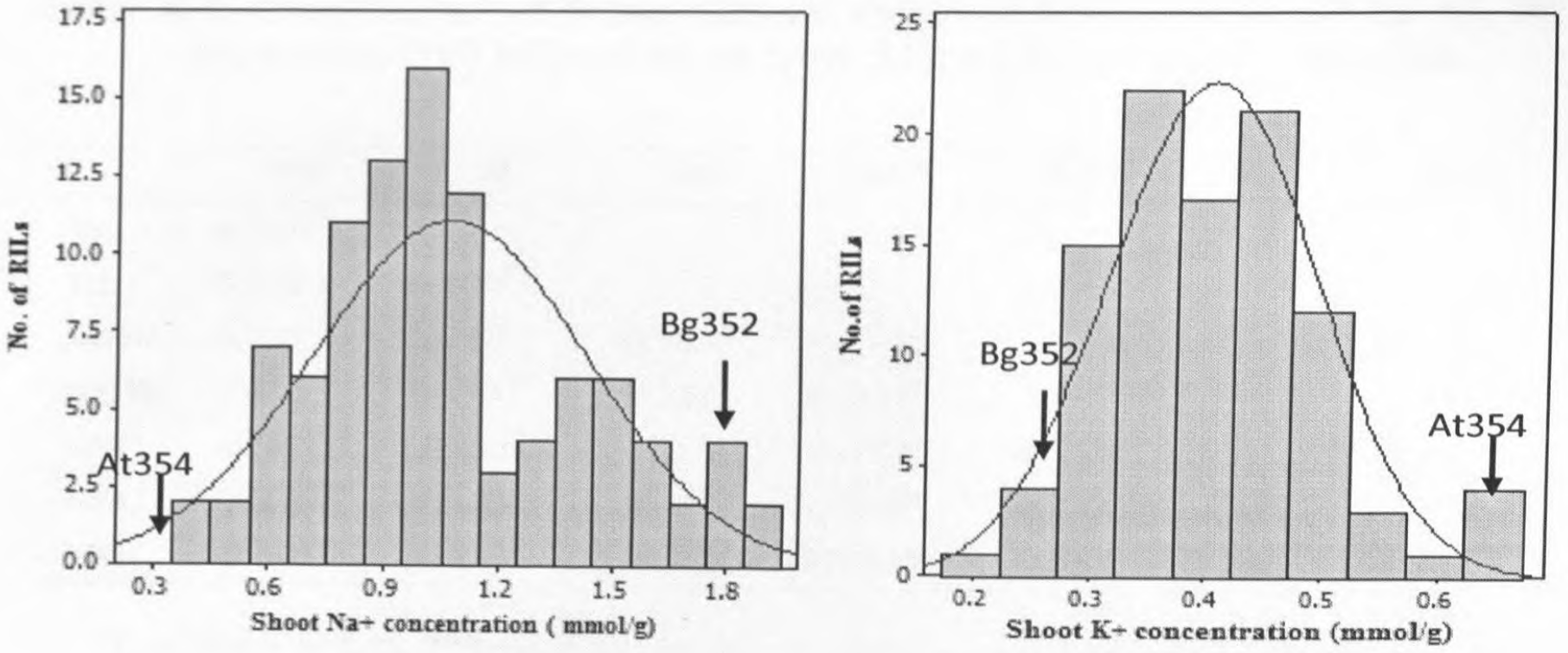


Figure 4.5. Frequency distributions of salinity survival index, shoot length, root length, root dry weight and shoot dry weight in RIL population derived from At354 x Bg352.
Parental means are indicated by arrow.



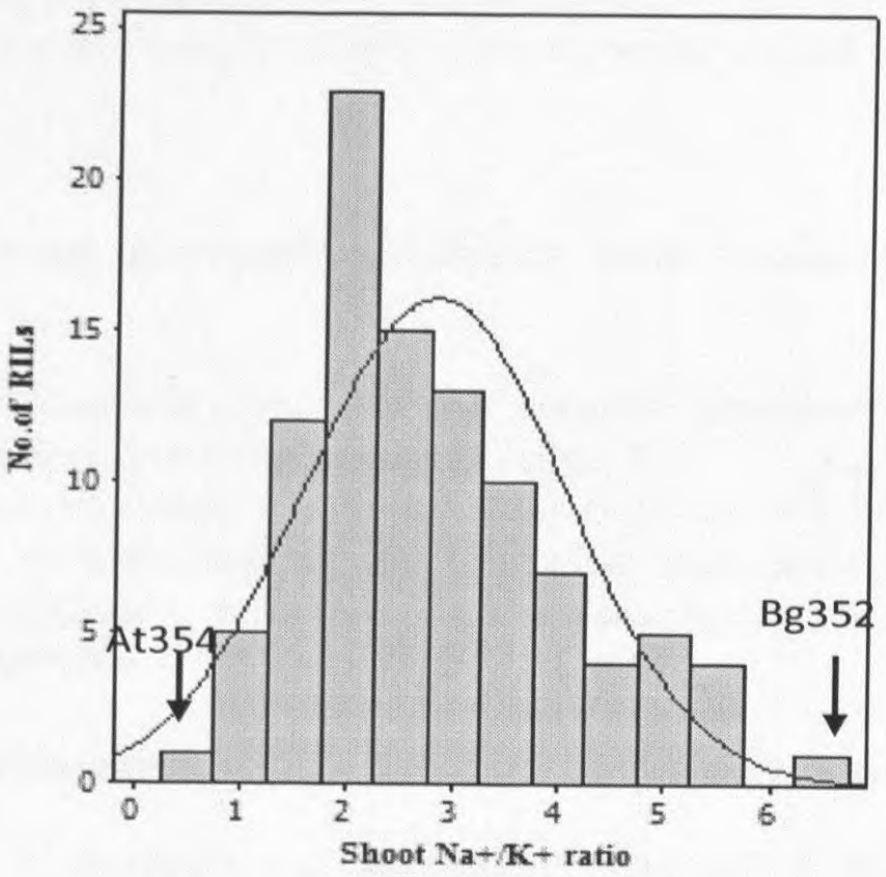


Figure 4.6. Frequency distributions of shoot Na^+ concentration, shoot K^+ concentration and shoot Na^+/K^+ ratio in RIL population derived from At354xBg352. Parental means are indicated by arrow.

Table 4.4. Correlations of 8 morpho-physiological traits examined in the RIL population (100 RILs) derived from At354 x Bg352 under salt stress

	SSI	SL	RL	SDW	RDW	SNC	SKC
SL	0.719^{***}						
RL	0.338_{**}	0.607^{***}					
SDW	0.246^{*}	0.242^{*}	0.168^{ns}				
RDW	0.430^{***}	0.359^{***}	0.244^{*}	0.719^{***}			
SNC	-0.367^{***}	-0.333^{**}	-0.230^{*}	-0.171^{ns}	-0.154^{ns}		
SKC	-0.275^{**}	-0.143^{ns}	0.057^{ns}	-0.040^{ns}	-0.129^{ns}	-0.196^{ns}	
SNK	-0.144^{ns}	-0.213^{*}	-0.205^{*}	-0.091^{ns}	-0.057^{ns}	0.849^{***}	-0.626^{***}

**Significant at 0.05, **Significant at 0.01, ***Significant at 0.001, ^{ns}Not significant*

Salinty Survival Index (SSI), Shoot Na^+ concentration (SNC), shoot K^+ concentration (SKC) and shoot Na^+/K^+ (SNK) ratio, Shoot length (SL), Root length(RL), Shoot fresh weight (SFW), Root fresh weight (RFW), Shoot dry weight (SDW), Root dry weight (RDW)

4.6. Polymorphism between parental genotypes with molecular markers in chromosome 1.

From the total of 57 SSR markers used for the parental polymorphism survey, 18 markers showed polymorphism between parents (Table 4.5) (Figure 4.7 and 4.8) of which 17 markers were located within the *Saltol* QTL region (10.8 Mb- 16.4 Mb) and only 1 marker, RM10287 was located at the telomeric region of the short-arm of chromosome 1 (5 Mb). Accordingly, selected SSR markers on chromosome 1 exhibited 31.57% of polymorphism between At354 and Bg352 parents.

4.7. Genotyping of RILs with polymorphic markers in chromosome 1

Out of 18 SSR markers, 12 markers *viz*. RM10852, RM10772, RM140, RM1287, RM493, RM10694, RM3412, RM10864, RM10793, RM10745, RM 10287and RM10711 which exhibited significant polymorphism between parental alleles were selected for the genotyping of RILs (Figure 4.7 and 4.8).

4.8. Construction of a linkage map in chromosome 1

Linkage map was constructed based on the genotypic data resulted with the 12 markers (Table 4.6) (Figure 4.9-4.11) using Kosambi mapping function. Accordingly, 12 markers were grouped into 2 linkage groups as seven markers in linkage group 1 and four markers in linkage group 2 (Figure 4.12). RM10793 was excluded from the linkage analysis as genotypic data produced for all 12 markers in the studied population were identical to genotypic data of RM10694. Linkage group 1 and 2 covers the narrow length of chromosome 1 as 46.9 cM and 44.0 cM respectively (Figure 4.12).

Table 4.5. Screening of 57 SSR markers located on chromosome 1 for polymorphism between At354 and Bg352 parents

Primer Name	Presence of polymophism (P/M)		Presence of polymophism (P/M)		Presence of polymophism (P/M)
RM8094*	P	RM562*	M	RM472	M
RM10764*	P	RM493*	P	RM10793*	P
RM10772*	P	RM8115*	M	RM11874	M
RM10745*	P	RM6711*	M	RM486	M
RM14	M	RM1287*	P	AP3206*	M
RM3412*	P	RM3825	M	RM3412b*	P
RM7075*	M	RM583	M	RM10720*	M
RM140*	P	RM10754*	M	RM10748*	P
RM243	M	RM1020	M	RM10843*	P
RM10852*	P	RM129	M	RM10800*	M
RM9*	M	RM10968 *	M	RM10871*	M
RM10710*	M	RM1329	M	RM10890*	M
RM10782*	M	RM10825*	M	RM10287	P
RM10746*	M	RM10864*	P	RM10927*	M
RM151	M	RM11757*	M	RM3252	M
RM1344	M	RM10694*	P	RM10115	M
RM10718*	M	RM7643	M	RM10701*	P
RM10776*	M	RM11008	M	RM10711*	P
RM581	M	RM11300	M	RM10829*	M

*SSR markers located within the *Saltol* region (10.8 Mb – 16. 4 Mb)
P- polymorphic markers between parents, M- Mormorphic markers between parents

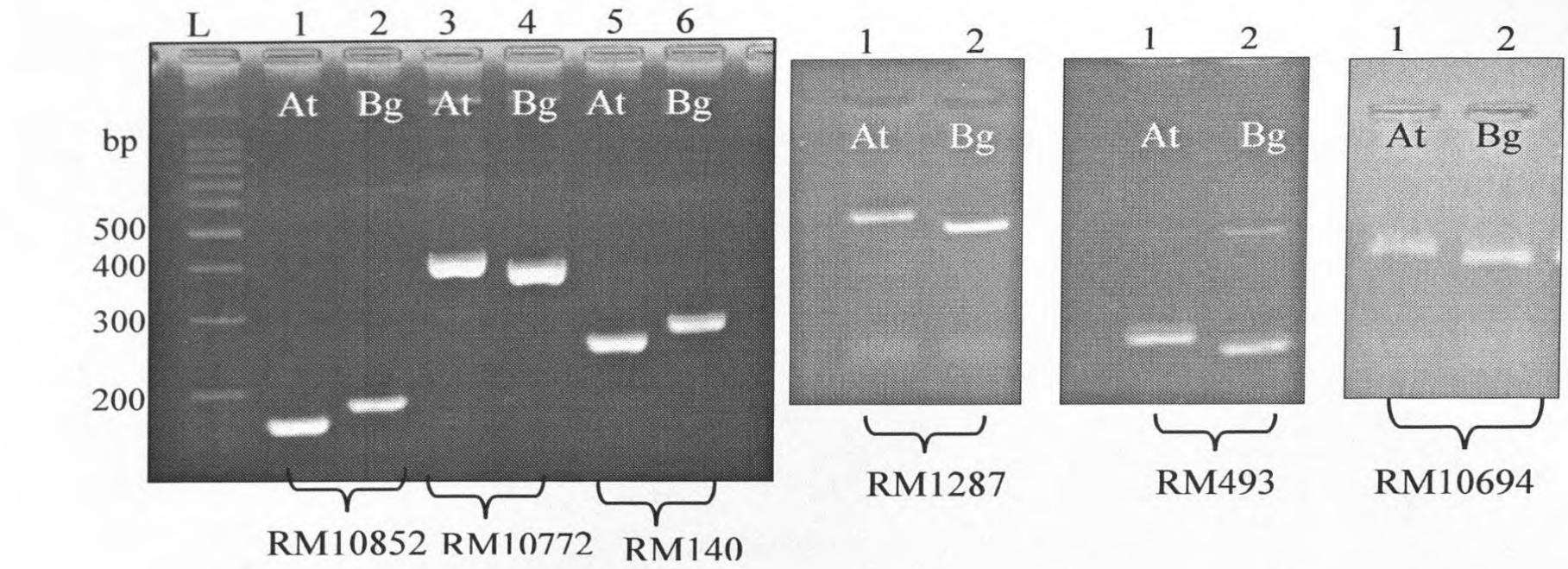


Figure 4.7. 3% agarose gel electrophoresis of PCR products resulted from the amplification of At354 and Bg352 parents with SSR markers RM10852, RM10772, RM140, RM1287, RM493, RM10694, RM8094, RM3412, RM10864 and RM10793

Lane L- 100 bp DNA ladder, At- At354 parent, Bg-Bg352 parent

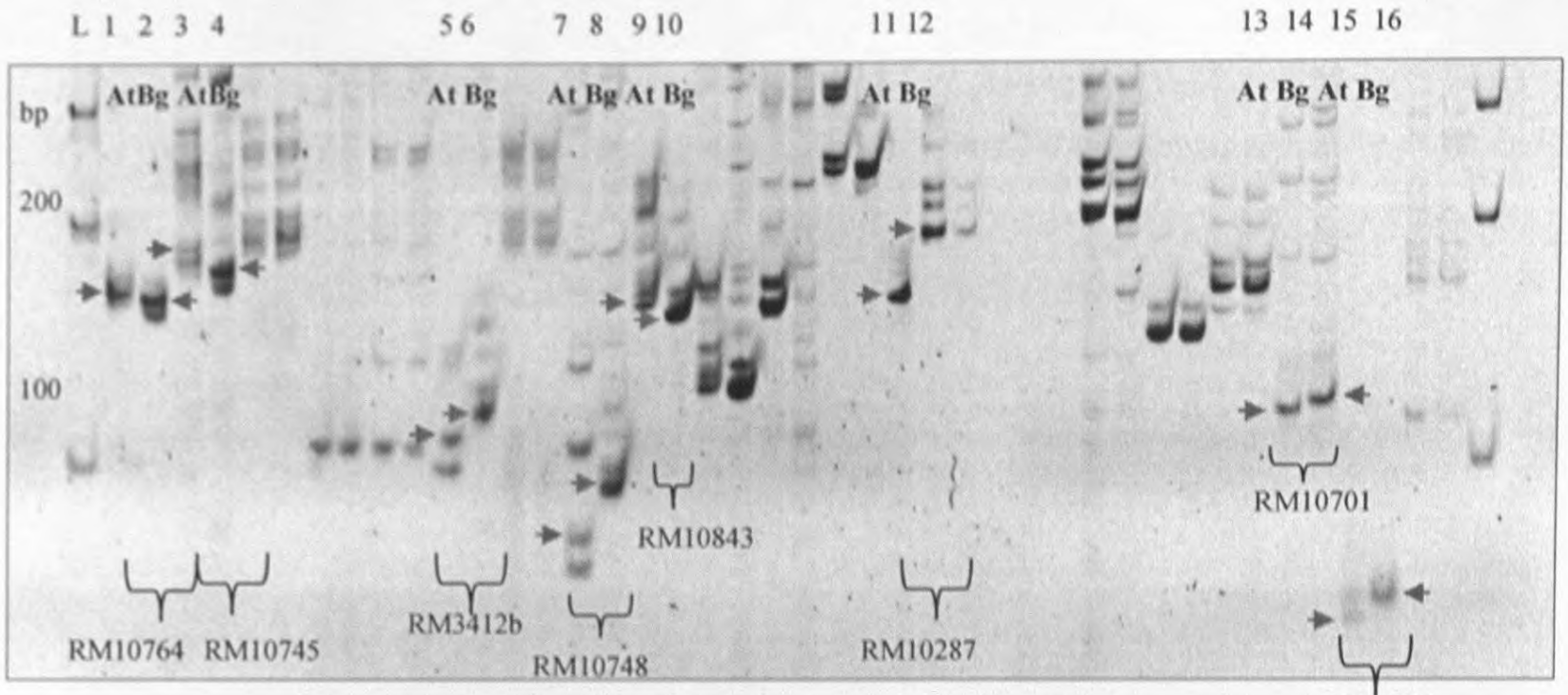


Figure 4.8. 8% Polyacrylamide gel electrophoresis of PCR products resulted from the amplification of At354 and Bg352 parents with SSR markers RM10764, RM10745, RM3412b, RM10748, RM104843, RM 10287, RM10701 and RM10711 SSR markers.

Lane L- 100 bp DNA ladder, At- At354 parent, Bg-Bg352 parent

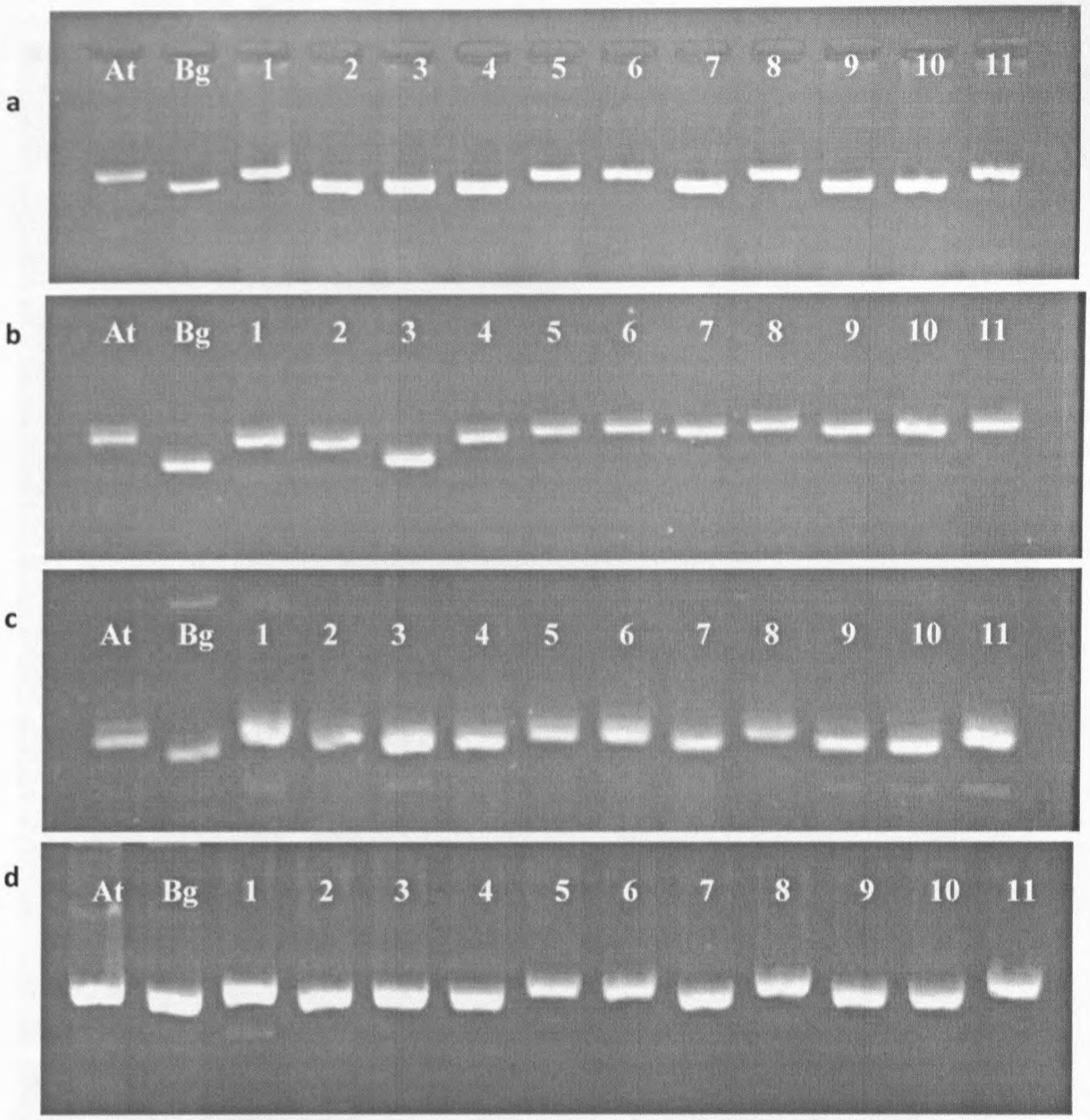


Figure 4.9. 3% agarose gel profile showing the amplification of 11 RILs along with At354 and Bg352 parents using SSR markers RM1287, RM10694, RM493 and RM10772

a- amplification with RM1287 SSR marker
b- amplification with RM10694 SSR marker
c- amplification with RM493 SSR marker
d- amplification with RM10772 SSR marker

At- At354 allele (upper fragment) Bg- Bg352 allele (lower fragment)
Lane 1-11- RIL No. 1,2,3,4,5,7,8,9,12,14 and 15

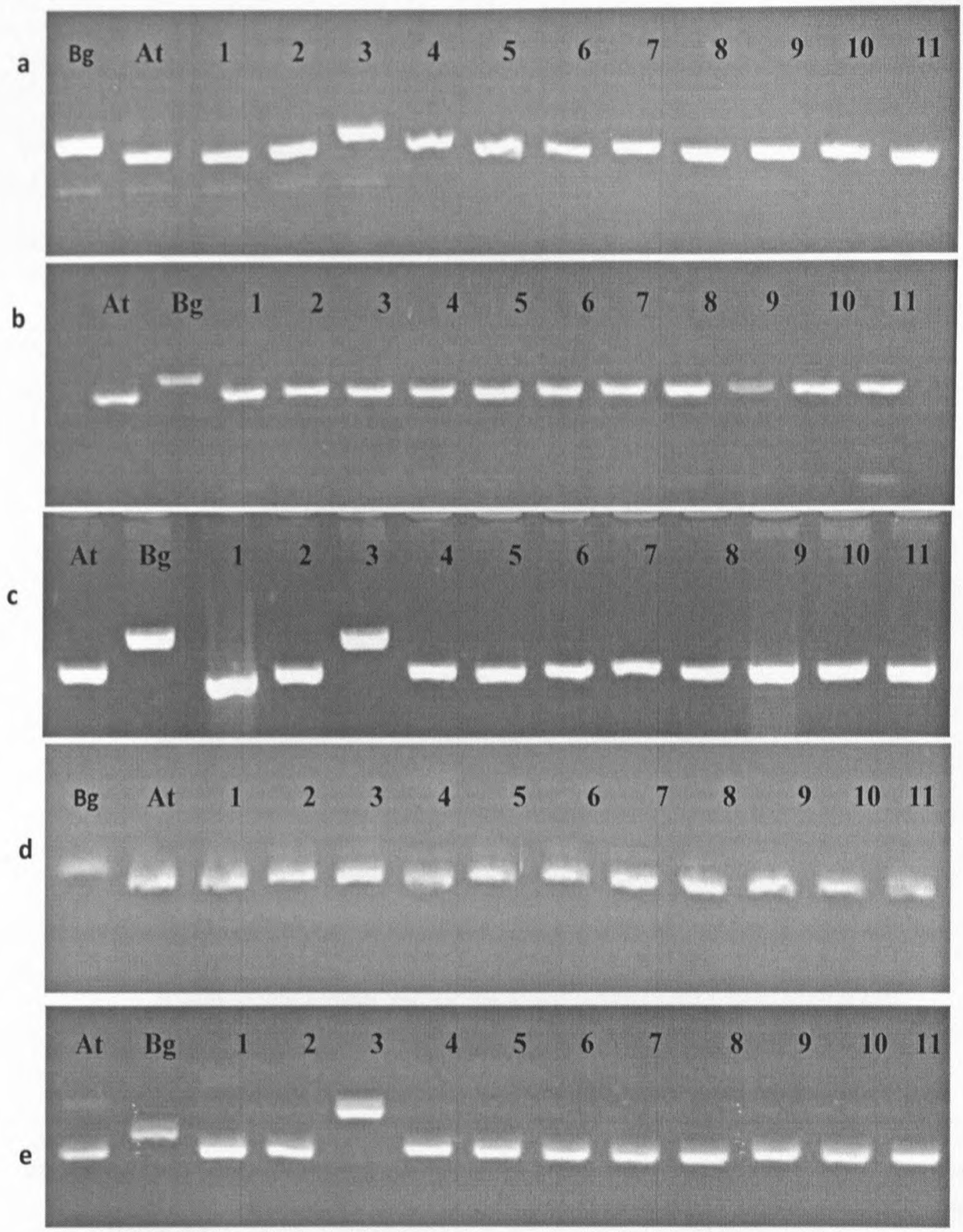


Figure 4.10. 3% agarose gel profile showing the amplification of 11 RILs along with At354 and Bg352 parents using SSR markers RM140, RM10852, RM10864 RM3412 and RM10793

a- amplification with RM140 SSR marker b- amplification with RM10852 SSR marker
c- amplification with RM10864 SSR marker d- amplification with RM3412 SSR marker
e-amplification with RM10793 SSR marker

At- At354 allele (lower fragment) Bg- Bg352 allele (upper fragment)
Lane 1-11- RIL No. 1,2,3,4,5,7,8,9,12,14 and 15

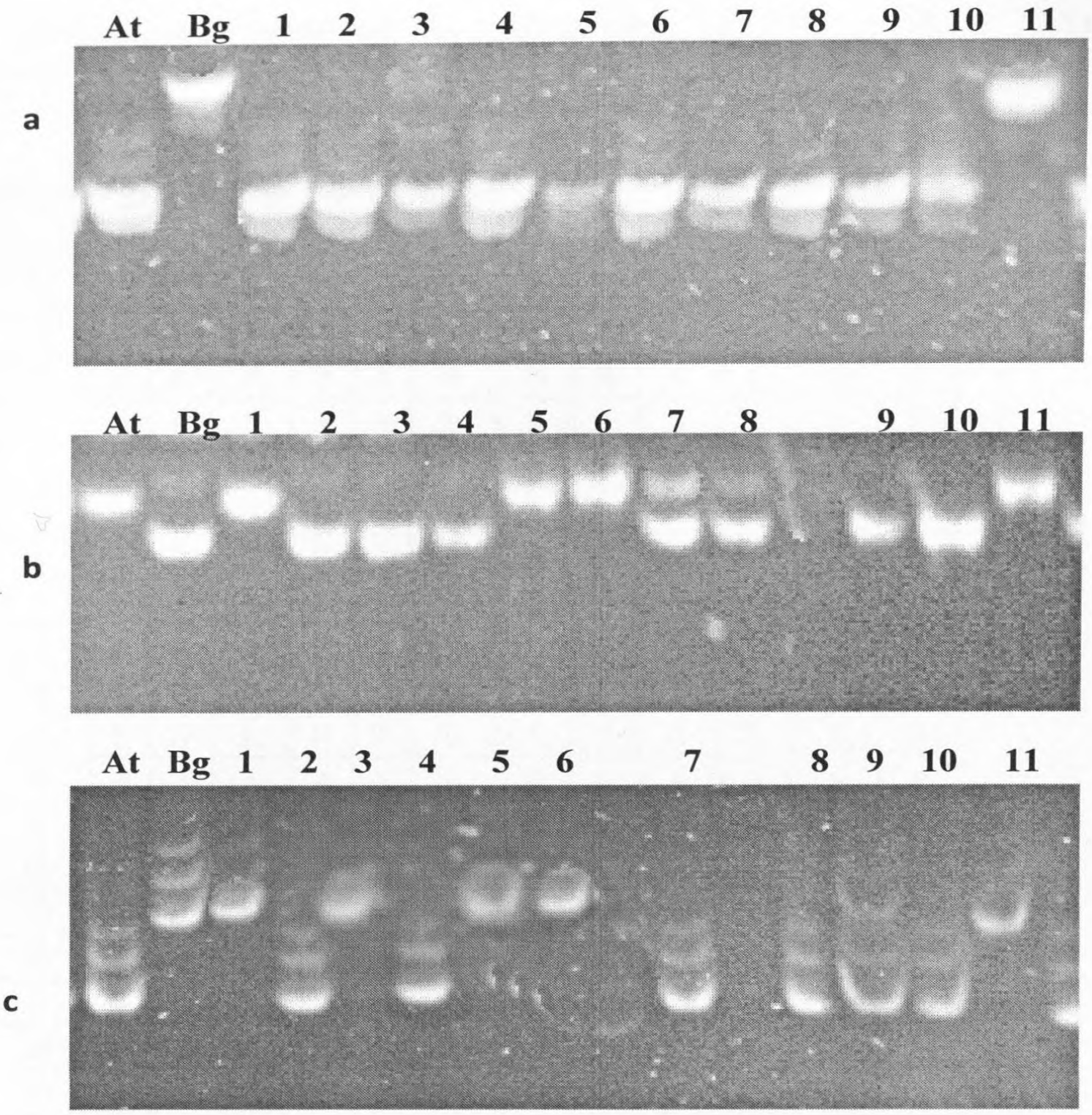


Figure 4.11. 8% polyacrylamide gel profile showing the amplification of 11 RILs along with At354 and Bg352 parents using SSR markers RM10287, RM10745 and RM10711

a- amplification with RM10287 SSR marker (At354 allele -lower fragment, Bg352 - allele upper fragment)

b- amplification with RM10745 SSR marker (At354 allele -upper fragment, Bg352 - allele lower fragment)

c- amplification with RM10711SSR marker (At354 allele -lower fragment, Bg352 - allele upper fragment)

At- At354 allele, Bg- Bg352 allele
Lane 1-11- RIL No. 1,2,3,4,5,7,8,9,12,14 and 15

Table 4.6.Genotypes obtained for RILs with 12 SSR markers located on chromosome 1

RIL No	RM10287	RM1287	RM10694	RM10711	RM3412	RM10745	RM10772	RM493	RM140	RM10793	RM10852	RM10864
1	A	A	A	B	A	A	A	A	A	A	A	A
2	A	B	A	A	A	B	B	A	A	A	A	A
3	A	B	B	B	A	B	B	A	B	B	A	B
4	A	B	A	A	A	B	B	A	B	A	A	A
5	A	A	A	B	A	A	A	A	B	A	A	A
7	A	A	A	B	A	A	A	A	B	A	A	A
8	A	B	A	A	A	h	B	A	B	A	A	A
9	A	A	A	A	A	h	A	A	A	A	A	A
12	A	B	A	A	A	B	B	A	A	A	A	A
14	A	B	A	A	A	B	B	A	A	A	A	A
15	B	A	A	B	A	A	A	A	A	A	A	A
16	A	A	A	A	A	B	B	B	B	A	A	A
17	A	B	A	A	A	B	B	B	A	A	A	A
18	A	A	A	B	A	A	A	B	A	A	A	A
19	A	A	A	B	A	A	A	B	A	A	A	A
20	A	B	A	A	A	B	B	B	A	A	A	A
21	A	B	B	-	B	h	B	B	B	B	B	B
22	A	A	A	-	A	A	A	B	A	A	A	A
23	B	A	A	-	A	A	A	B	A	A	A	A
24	A	B	A	A	A	B	B	B	B	A	A	A
25	A	B	A	A	A	B	B	B	B	A	A	A
29	A	B	A	A	A	B	A	h	A	A	A	A
30	A	A	A	-	A	A	A	B	A	A	A	A
31	A	A	A	A	A	B	A	B	B	A	A	A
32	A	A	A	A	A	h	A	A	A	A	A	A
33	A	A	A	A	A	h	A	A	A	A	A	A
34	h	A	A	B	A	h	A	A	A	A	A	A
36	A	B	A	A	A	B	B	B	A	A	A	A
37	A	B	A	A	A	B	B	B	A	A	A	A
40	B	A	A	B	A	B	A	A	A	A	A	A
42	B	B	B	B	B	B	B	B	B	B	B	B
43	A	A	A	B	A	A	A	A	A	A	A	A
44	A	A	A	B	A	A	A	A	A	A	A	A

RIL No	RM10287	RM1287	RM10694	RM10711	RM3412	RM10745	RM10772	RM493	RM140	RM10793	RM10852	RM10864
45	A	B	A	A	A	B	B	B	A	A	A	A
47	A	B	B	B	A	B	B	B	B	B	A	B
50	A	B	A	A	A	h	B	B	A	A	A	A
51	A	A	A	B	A	A	A	A	A	A	A	A
53	A	B	A	A	A	B	B	B	A	A	A	A
54	A	B	A	A	A	B	B	B	A	A	A	A
56	A	B	A	A	A	B	B	B	A	A	A	A
58	A	B	A	A	A	B	B	B	A	A	A	A
59	A	B	A	A	A	B	B	B	A	A	A	A
61	A	B	A	A	A	B	B	B	A	A	A	A
64	A	A	A	B	A	A	A	A	A	A	A	A
66	A	B	A	A	A	B	B	B	A	A	A	A
71	A	A	A	B	A	A	B	A	A	A	A	A
72	A	A	A	B	A	A	B	A	A	A	A	A
74	A	B	A	A	A	B	B	B	A	A	A	A
76	A	A	A	B	A	A	A	B	A	A	A	A
77	A	A	A	A	A	B	A	B	A	A	A	A
78	A	B	A	A	A	B	B	B	A	A	A	A
80	A	h	A	A	A	h	A	h	A	A	A	A
82	A	A	A	B	A	A	A	B	A	A	A	A
83	A	A	A	B	A	A	A	B	A	A	A	A
84	A	A	A	A	A	B	A	B	A	A	A	A
85	A	B	A	A	A	h	B	B	A	A	A	A
87	A	A	A	B	A	A	B	B	A	A	A	A
89	A	B	A	A	A	B	B	B	A	A	A	A
90	A	B	A	A	A	B	B	B	A	A	A	A
95	A	A	A	B	A	A	A	B	A	A	A	A
96	A	B	A	A	A	B	B	B	A	A	A	A
97	B	A	A	-	A	A	A	A	A	A	A	A
98	A	A	A	A	A	B	A	A	A	A	A	A
99	A	A	A	B	A	A	A	A	A	A	A	A
100	A	B	A	A	A	B	B	B	A	A	A	A
102	A	h	A	B	A	A	A	h	A	A	A	A

RIL No	RM10287	RM1287	RM10694	RM10711	RM3412	RM10745	RM10772	RM493	RM140	RM10793	RM10852	RM10864
103	A	A	A	B	A	A	A	A	A	A	A	A
105	A	h	A	A	A	h	A	h	A	A	A	A
106A	A	h	A	A	A	A	A	A	A	A	A	A
106	A	B	A	A	A	B	B	B	A	A	A	A
107	A	B	A	A	A	B	B	B	A	A	A	A
109	A	A	A	B	A	A	B	A	A	A	A	A
110	A	A	A	A	A	B	B	B	A	A	A	A
111	A	B	A	A	A	B	B	B	A	A	A	A
113	A	B	A	A	A	B	B	B	A	A	A	A
114	A	B	A	A	A	B	B	B	A	A	A	A
117	A	B	A	A	A	B	B	B	A	A	A	A
118	A	A	A	-	A	A	A	A	A	A	A	A
119	A	B	A	A	A	B	B	B	A	A	A	A
121	A	B	A	A	A	B	B	B	A	A	A	A
123	A	A	A	B	A	A	A	A	A	A	A	A
124	A	A	A	B	A	A	A	A	A	A	A	A
125	A	A	A	A	A	B	B	B	A	A	A	A
134	A	A	A	B	A	A	A	A	A	A	A	A
137	A	B	A	B	A	A	A	B	A	A	A	A
138	A	h	A	B	A	A	h	h	A	A	A	A
145	A	B	A	A	A	B	B	B	A	A	A	A
149	A	B	A	A	A	A	B	B	A	A	A	A
150	A	B	A	A	A	B	B	B	A	A	A	A
151	A	A	A	B	A	A	A	A	A	A	A	A
153	A	B	A	A	A	B	B	B	A	A	A	A
154	A	A	A	A	A	A	A	A	A	A	A	A
155	A	B	A	A	A	B	A	A	A	A	A	A
158	B	B	h	A	h	B	B	h	h	h	B	B
159	A	A	A	-	A	A	A	B	A	A	A	A
162	A	B	A	-	B	B	B	A	A	A	B	B

A- At354 allele, B-Bg352 allele, h- heterozygous, "-" missing data

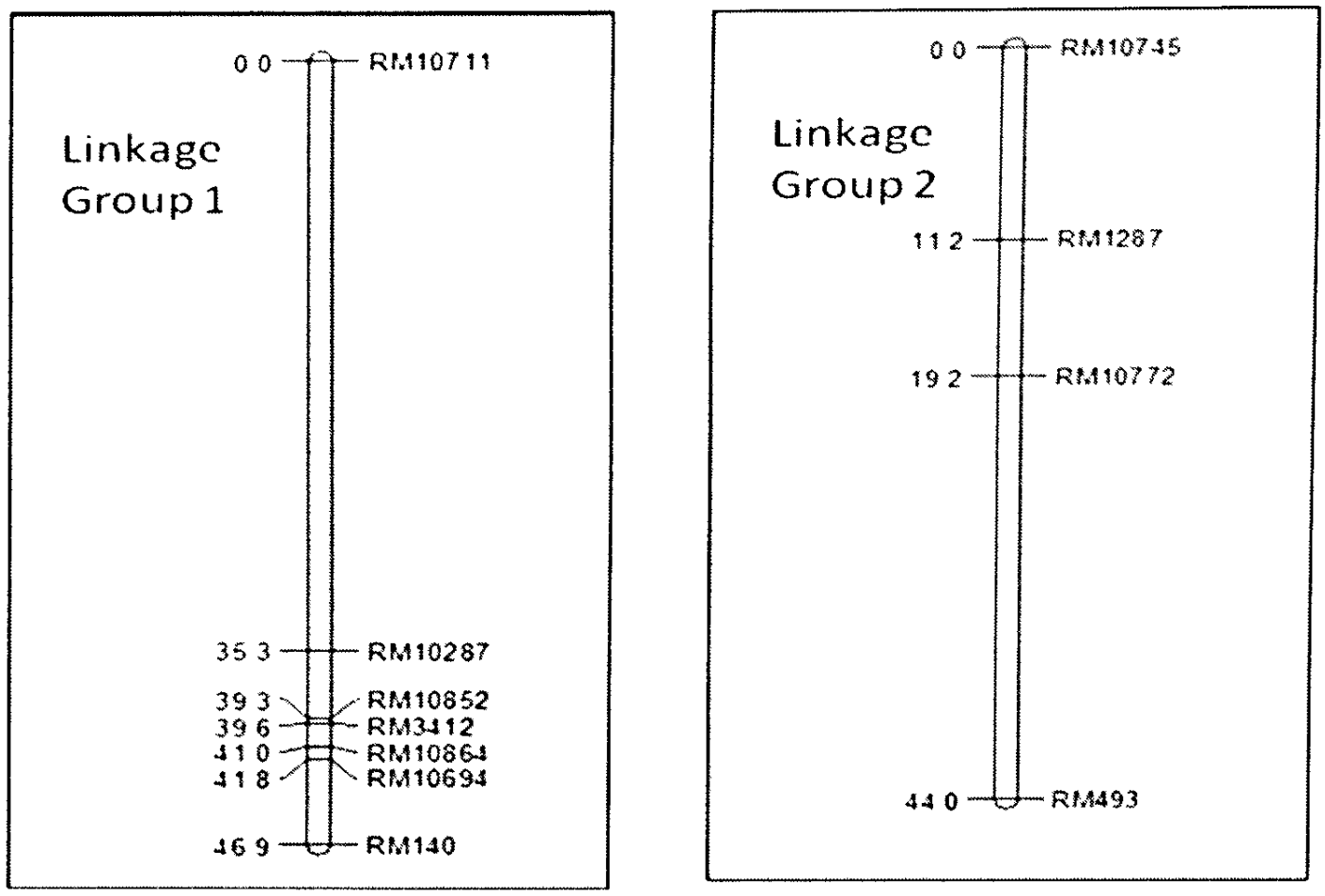


Figure 4.12. Linkage map of chromosome 1 showing 2 linkage groups resulted from 11 polymorphic SSR markers.
Distances are indicated in Kosambi centimorgans

4.9. Detection of QTLs in chromosome 1

4.9.1. Detection of QTLs by single marker analysis method.

Using single marker analysis (student's t-test), 12 SSR marker loci were analyzed to detect whether there is an association between marker locus and the 5 quantitative traits assessed in this study (Table 4.7). Accordingly, significant differences were observed between At354 and Bg352 marker genotypes at RM140 locus with SSI ($p<0.001$), shoot length ($p<0.01$) and root length ($p<0.05$) indicating the possibility of having association of these 3 traits at RM140 locus (Table 4.7). Both shoot Na^+/K^+ ratio and shoot K^+ concentration exhibited significant differences between At354 and Bg352 genotypes at RM1287 ($p<0.05$) and RM10745 ($p<0.001$) inferring probable association at these 2 loci. Other than these two loci, shoot Na^+/K^+ ratio was also significantly associated with RM10772 ($p<0.05$) whereas shoot K^+ concentration exhibited association with RM493 and RM10711 at 0.01 significance level.

Moreover, at RM10745 marker locus, shoot Na^+ concentration was exhibited significant difference between parental genotypes at 0.05 significance level. It was noted that among the 12 marker loci, RM140 marker locus was significantly associated with 3 traits i.e. Shoot length, root length and SSI while RM10745 was significantly associated with shoot Na^+/K^+ ratio, shoot Na^+ concentration and shoot K^+ concentration. Furthermore, it is interesting to note that all these associations were detected within the previously reported *Saltol* QTL region.

4.9.2. Mapping of QTLs by single marker regression.

Each marker locus was analyzed by single marker regression (SMR) method which is similar to student's t-test using Qgene 4.3.10 software. Accordingly, 4 significant

putative QTLs for SSI, shoot length, shoot Na^+/K^+ ratio and shoot K^+ concentration were detected with LOD score ranging from 2.9-3.1 explaining 11.8-14.2% phenotypic variation of each trait (Table 4.8). All 4 QTL positions were significant at 0.01 level of significance according to the LOD threshold resulted with 1000 permutation. Out of 4 QTLs, QTLs responsible for SSI and shoot length were co-localized at the RM140 SSR marker flanking RM10772 and RM10793 markers at 12.3 Mb position (Table 4.8)) (Figure 4.13 a and b). Co-localization of rest of the 2 QTLs responsible for shoot Na^+/K^+ ratio and shoot K^+ concentration was also observed at RM10745 SSR marker flanking RM3412 and RM10772 at 11.7 Mb position (Figure 4.13 c and d). These results were consistent with the results of the student's t-test in which highly significant association ($p<0.001$) was detected at RM140 (SSI and shoot length) and RM10745 for (shoot Na^+/K^+ ratio and shoot K^+ concentration). However, rest of the significant associations identified by student's t-test was not detected by single marker regression method.

4.9.3. Mapping of QTLs by composite interval mapping.

Moreover, genotypic and phenotypic data were analyzed by composite interval mapping (CIM) which combines interval mapping with linear regression to include more markers as cofactors, in order to remove the effects of multiple QTLs and enhance the power of detection of QTLs (Zeng, 1994; Collard et al., 2005). Accordingly, only 3 significant major QTLs were detected for SSI (*qSSI1*), shoot length (*qSL1*) and shoot Na^+/K^+ ratio (*qSNK1*) (Table 4.8) (Figure 4.14-4.16). Putative QTL identified for shoot K^+ concentration by both student's t-test and SMR was not significant with CIM analysis. It was noted that all 3 QTLs were associated with the comparatively low LOD value ranging from 1.95 to 2.38 which were significant at mainly 0.05 significance level and accounting 8.9 to 10.8% phenotypic variability (Table 4.8).

qSSI1 and qSL1 QTL positions were slightly shifted from 12.3 to 12.5 Mb position and coincided with the RM10793 SSR marker indicating certain deviation from the results of the single marker analysis (Figure 4.14 and 4.15). Similarly *qSNK1* QTL was moved to 12 Mb position and located closer to the RM10772 SSR marker (Figure 4.16). Further, positive additive effect was observed at *qSSI1* and *qSL1* QTL positions indicating the contribution of At354 alleles to increase the traits by 0.146 and 6.711 cm respectively (Table 4.8) (Figure 4.14 and 4.15). Bg352 alleles have contributed to enhance the effect of shoot Na^+/K^+ ratio at *qSNK1* QTL by 0.391 (Table 4.8) (Figure 4.16). However, as salinity tolerance is mainly attributed with low shoot Na/K ratio, At354 could be considered as the donor of salt tolerant allele at *qSNK1* QTL. Summary of the locations of the reported QTLs in chromosome 1 are indicated in the figure 4.17.

Table 4.7. Probability values resulted from the single marker analysis (student's t-test) in chromosome 1

Trait[a]	RM10694	RM1287	RM3412	RM10772	RM493	RM140	RM10793	RM10852	RM10864	RM10287	RM10745	RM10711
RL	0.227	0.953	0.964	0.935	0.049	**0.029***	0.109	0.843	0.268	0.338	0.606	0.700
SL	0.319	0.235	0.864	0.966	0.200	**0.001****	0.214	0.706	0.225	0.609	0.475	0.196
RDW	0.540	0.378	0.370	0.890	0.240	0.047	0.288	0.423	0.195	0.706	0.627	0.954
SDW	0.966	0.214	0.829	0.261	0.329	0.439	0.785	0.870	0.722	0.816	0.723	0.136
SSI	0.229	0.742	0.874	0.241	0.076	**0.000*****	0.263	0.679	1.000	0.474	0.587	0.683
SNK	0.370	**0.016***	0.234	**0.010***	0.173	0.638	0.370	0.129	0.944	0.096	**0.000*****	0.068
SNC	0.412	0.228	0.212	0.081	0.630	0.045	0.412	0.102	0.898	0.118	**0.027***	0.578
SKC	0.745	**0.016***	0.485	0.052	**0.006****	0.068	0.745	0.504	0.886	0.119	**0.000*****	**0.007****

[a]Root length(RL), Shoot length (SL), Root dry weight (RDW), Shoot dry weight (SDW), Salinty Survival Index (SSI), Shoot Na^+ concentration (SNC), Shoot K^+ concentration (SKC) and Shoot Na^+/K^+ (SNK) ratio,

**Significant at 0.05, **Significant at 0.01, ***Significant at 0.001, [ns]Not significant.*

Table 4.8. Identified putative QTLs for 4 morpho-physiological traits assessed in RIL population derived from At354/Bg352 cross under salinity stress

QTL analysis method[a]	Trait[b]	QTL	Chr	Peak marker	Flanking markers		Peak position (cM)	Peak Position (Mb)	Add. effect[c]	LOD	R^2 (%)	LOD threshold		Salt tolerant allele Donor[d]
												α = 0.05	α = 0.01	
SMR	SSI	*qSSI1*	1	RM140	RM10772	RM10793	49.2	12.3	0.122	3.196	14.2	1.641	2.55	At354
	SL	*qSL1*	1	RM140	RM10772	RM10793	49.2	12.3	3.042	2.614	11.8	1.718	2.328	At354
	SNK	*qSNK1*	1	RM10745	RM3412	RM10772	46.8	11.7	-0.477	2.921	13.1	1.809	2.697	At354
	SKC	*qSKC1*	1	RM10745	RM3412	RM10772	46.8	11.7	0.035	2.953	13.2	1.692	2.573	At354
CIM	SSI	*qSSI1*	1	RM10793	RM140		50	12.5	0.146	2.385	10.8	1.536	2.319	At354
	SL	*qSL1*	1	RM10793	RM140		50	12.5	6.711	2.206	10	1.643	2.395	At354
	SNK	*qSNK1*	1	RM10772	RM10745	RM140	48	12	-0.391	1.935	8.9	1.595	2.378	At354

[a] SMR - Single marker regression, CMI- Composite interval mapping

[b]Salinity survival index (SSI), Shoot length (SL), Shoot Na+ concentration (SNC), Shoot K+ concentration(SKC) and Shoot Na+/K+(SNK) ratio

Chr - chrchromosome

[c] positive value means At354 alleles contribute to increase effect of respective trait, negative value means Bg352 alleles contribute to increase effect of respective trait

[d]Allele donor in favour of salt tolerance

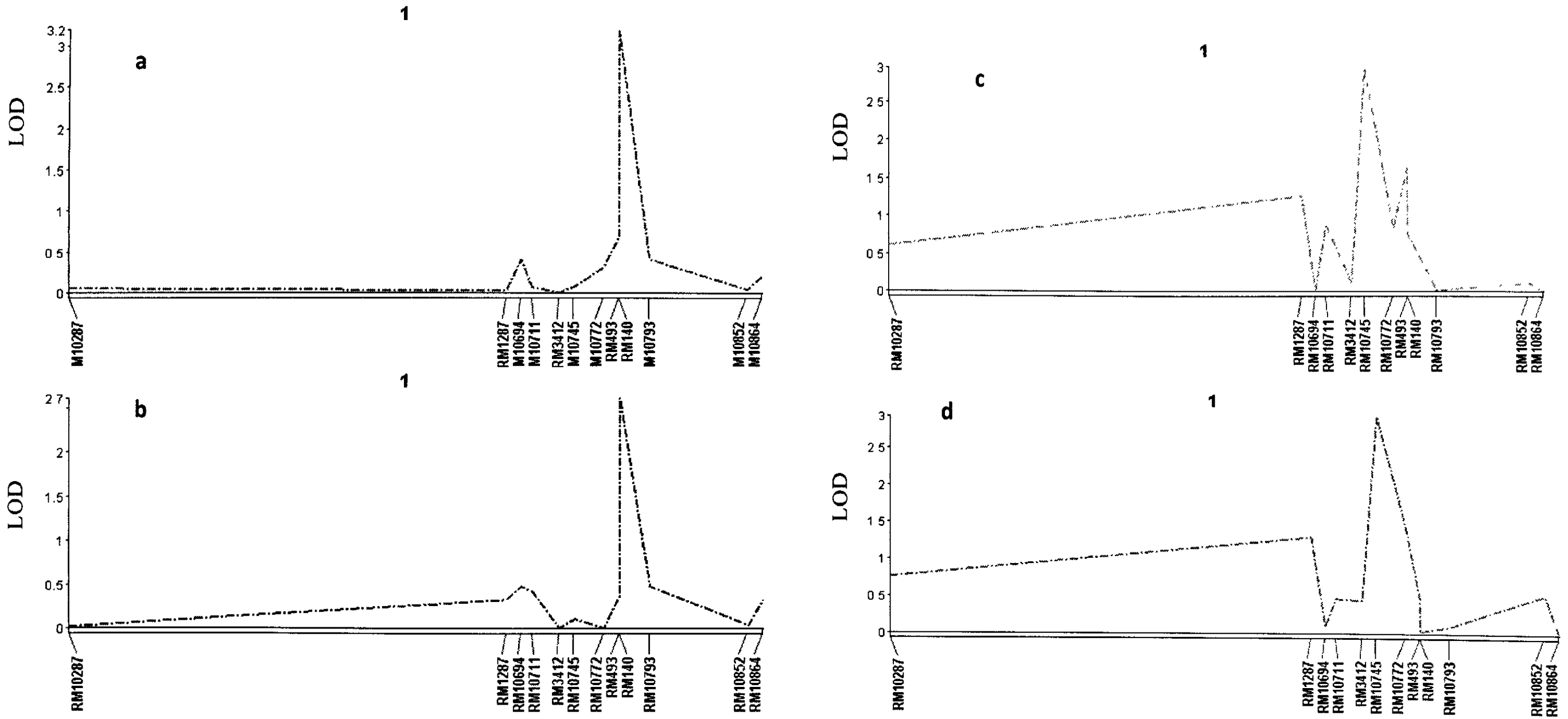


Figure 4.13. QTL maps of salinity survival index, shoot length, shoot K^+ concentration and shoot Na^+/K^+ ratio on chromosome 1 resulted by single marker regression (SMR).

a- QTL map of salinity survival index b- QTL map of shoot length c- QTL map of shoot K^+ concentration

d- QTL map of shoot Na^+/K^+ ratio

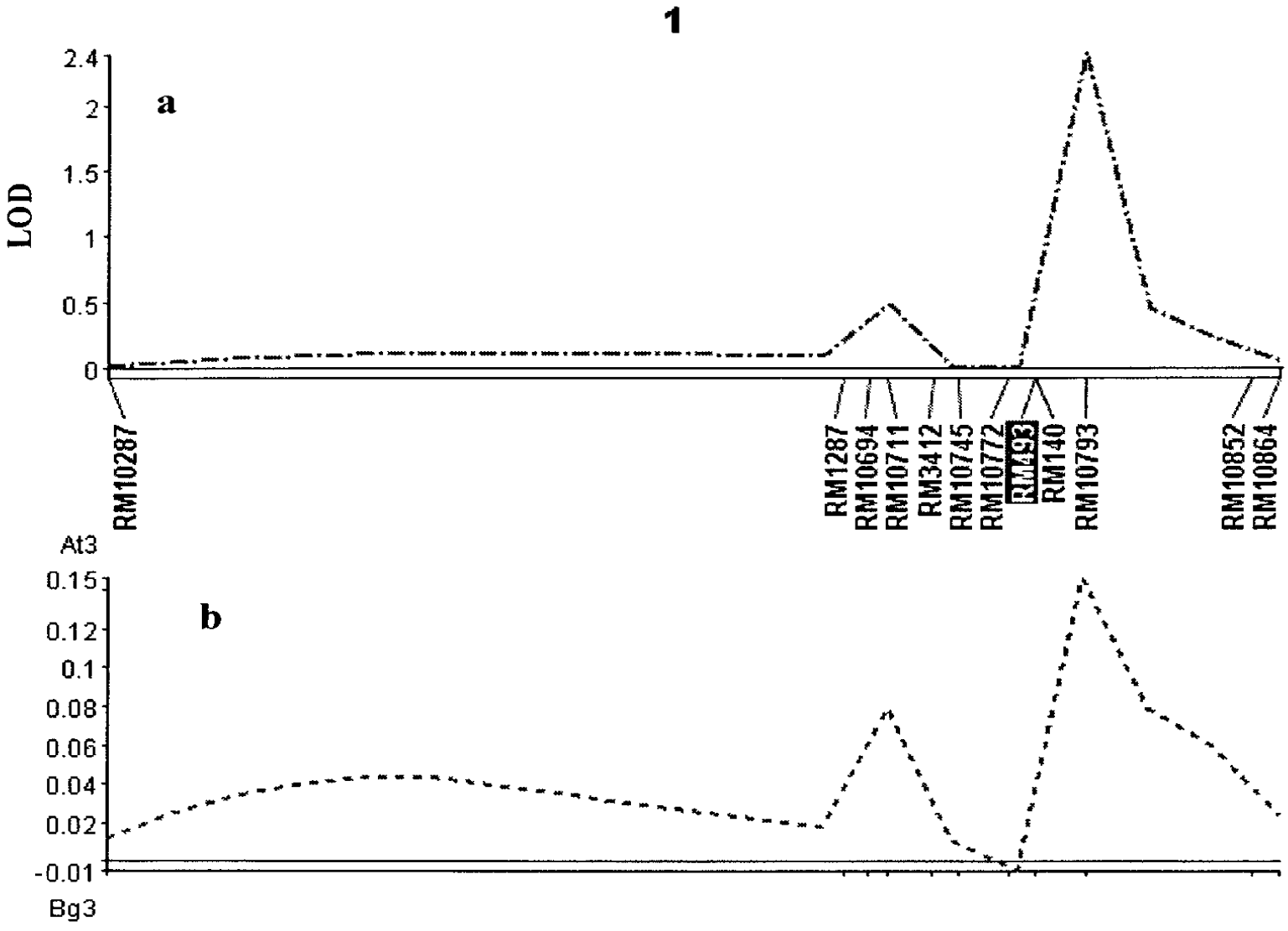


Figure 4.14. QTL map and additive effect graph of salinity survival index on chromosome 1 resulted by composite interval mapping (CIM)

a- QTL map of salinity survival index showing the location of *qSSI1*
b- Graph of additive effect indicating contribution of At354 and Bg352 alleles for salinity survival index.

At3- At354, Bg3- Bg352

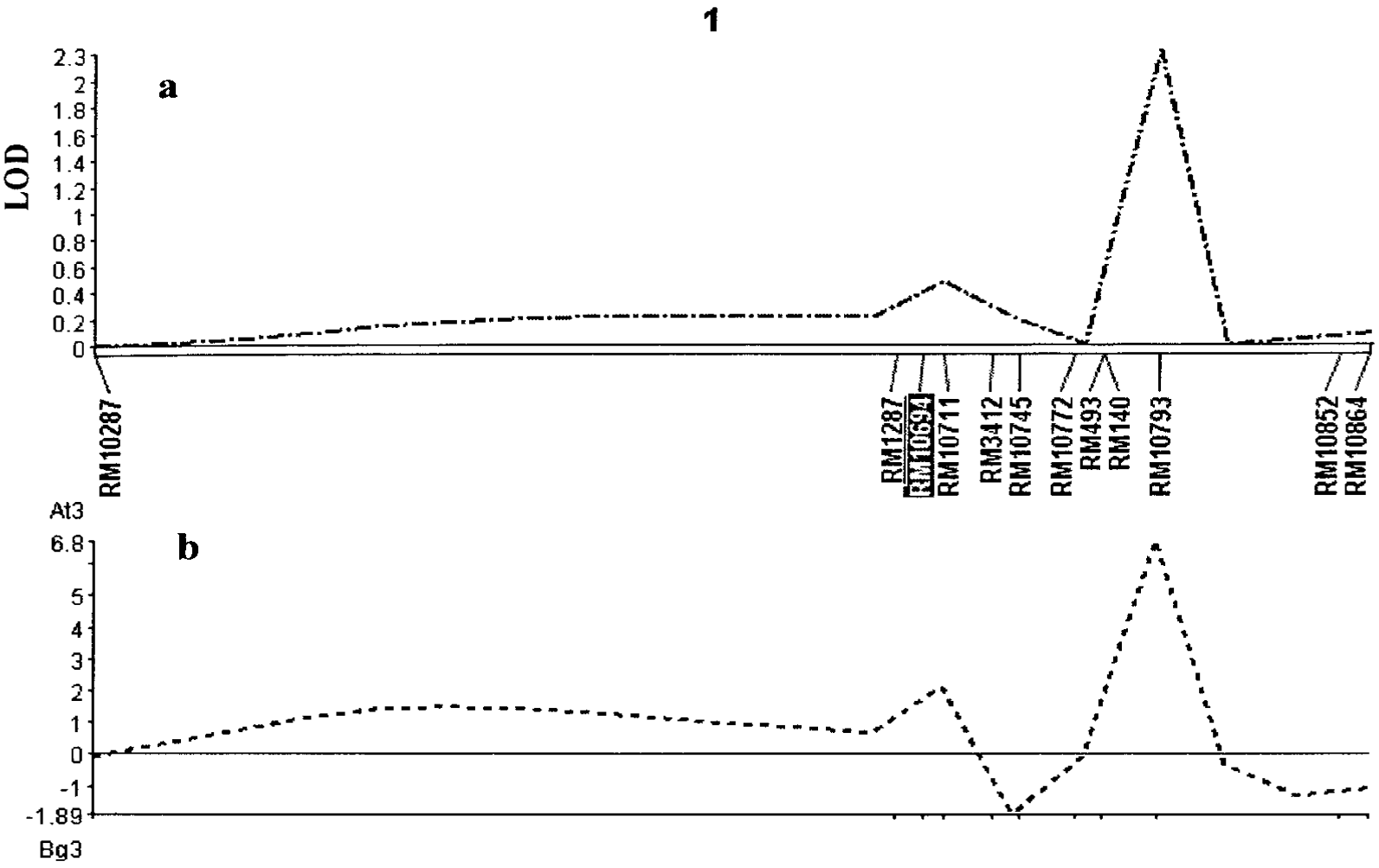


Figure 4.15. QTL map and additive effect graph of shoot length on chromosome 1 resulted by composite interval mapping (CIM)

a- QTL map of shoot length showing the location of *qSL1*
b- Graph of additive effect indicating contribution of At354 and Bg352 alleles for shoot length.

At3- At354, Bg3- Bg352

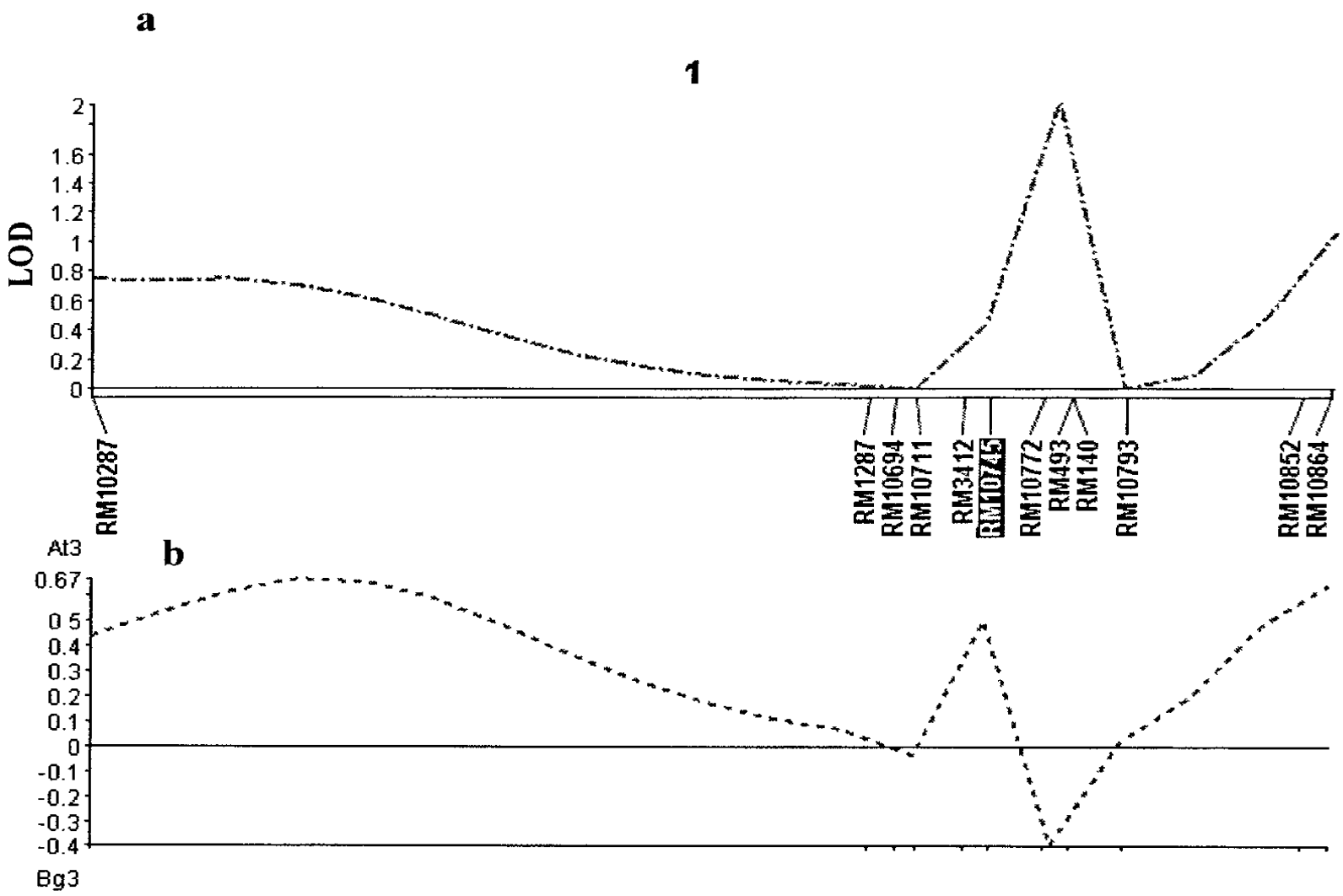


Figure 4.16. QTL map and additive effect graph of shoot Na^+/K^+ ratio on chromosome 1 resulted by composite interval mapping (CIM)

a- QTL map of shoot Na^+/K^+ ratio showing the location of *qSNK1*
b- Graph of additive effect indicating contribution of At354 and Bg352 alleles for shoot Na^+/K^+ ratio.

At3- At354, Bg3- Bg352

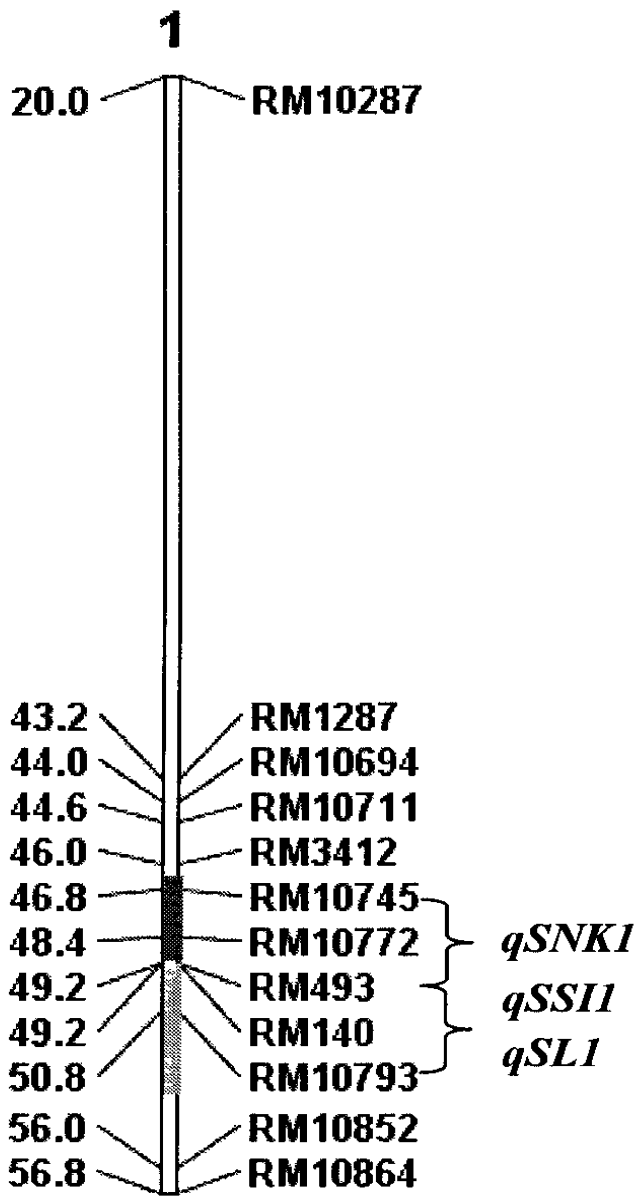


Figure 4.17. Putative QTLs identified on chromosome 1 using composite interval mapping (CIM). Distances are indicated in Kosambi centimorgans.

qSSI1 – QTL identified for salinity survival index
qSL1- QTL identified for shoot length
qSNK1 - QTL identified for shoot Na+/K+ ratio

4.10. Polymorphism between parental genotypes with molecular markers in chromosome 3

In 3% percent agarose electrophoresis polymorphism was detected in 5 SSR markers (Figure 4.18) out of 22 in chromosome 3 between parents (Table 4.9). Eight SSR markers, which did not show polymorphism in agarose, were analyzed under urea-denaturing Polyacrylamide gel electrophoresis (PAGE). Figure 4.19 showed that only two polymorphic markers could be identified in PAGE. The percentage of polymorphic markers found in chromosome 3 was 31.8% (Table 4.22).

4.11. Genotyping of RILs with polymorphic markers in chromosome 3.

The RIL population was genotyped with five SSR markers i.e. RM7, RM5626, RM251, RM6326 and RM36 which showed polymorphism in agarose gel electrophoresis (Figure 4.20). There were 85 individuals showing At354 parent allele and 11 individuals showing Bg352 allele observed for RM7 marker and RIL population was 100% homozygous for RM7. For RM 5626 marker, RILs were 97.9% homozygous and there were 63 lines showed At354 allele, 31 lines showed Bg352 and 2 lines showed both the parental alleles. The number of RILs exhibited At354 allele and Bg352 allele for the marker RM6326 was 82 and 11 respectively. There were 3 lines which were heterozygous for RM6326. Therefore RILs were 96.8% homozygous for RM6326. For RM251 and RM36 markers there were 85 and 74 individuals showing At354 allele and 10 and 22 individuals showing Bg352 allele observed respectively. RIL population was 100% homozygous for both the markers. (Table 4.10).

4.12. Construction of linkage map and identification of QTLs in chromosome 3

Linkage map of the chromosome 3 was constructed with the genotypic data obtained from the amplification of polymorphic markers. All the five markers were included in one linkage group and the markers were distributed throughout the chromosome with an average of 12.52 cM linkage distance (Figure 4.21). There was a 26 cM linkage distance between markers RM36 and RM6329, a 4.7 cM distance was observed for RM6329 and RM7. The smallest distance 0.6 cM was observed between RM7 and RM251. RM5626 was located 30.6 cM away from RM251. Markers RM6329, RM7 and RM251 have concentrated towards the centre region of the chromosome with less distance in the linkage map of chromosome 3.

Single marker analysis, revealed that none of the markers were significantly associated with phenotypic traits indicating the absence of QTLs in chromosome 3 for measured phenotypic traits (Table 4.11). The same results were obtained by the composite interval mapping method (Figure 4.22), indicating absence of salinity tolerant QTLs in chromosome 3.

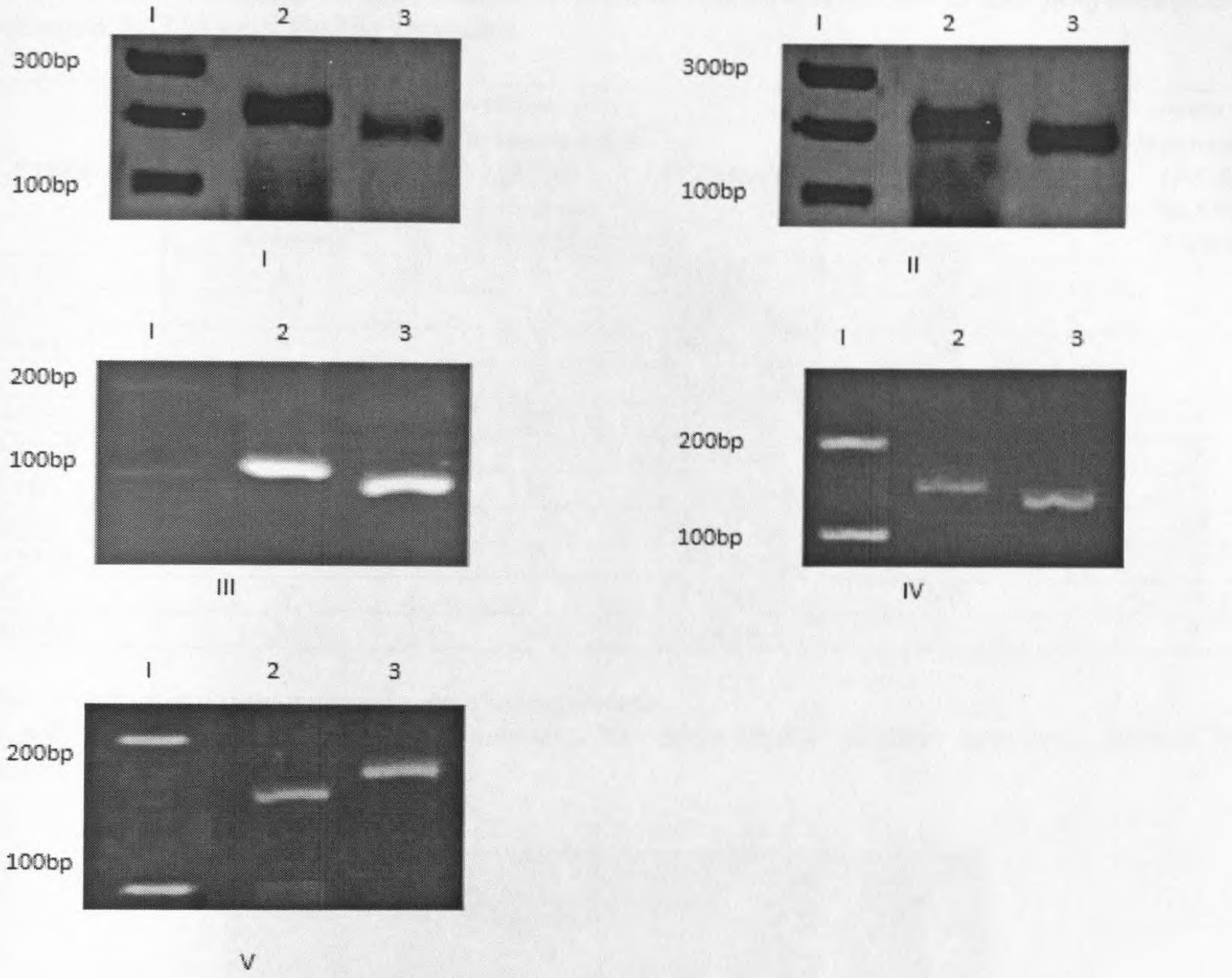


Figure 4.18. PCR amplification with RM251, RM6329, RM5626, RM7 and RM36 SSR markers located on chromosome 3, showing polymorphism between At354 and Bg352 parental lines

I) RM251, (II) RM6329, (III) RM5626, (IV) RM7 and (V) RM36

Lane 1- 100bp DNA ladder, Lane 2- At354, Lane 3- Bg352

Table 4.9. Screening of SSR markers located on chromosome 3 for polymorphism between At354 and Bg352 parents.

Primer	Presence of polymorphism (P/M) in 3% Agarose	Presence of polymorphism (P/M) in 6% PAGE	Primer	Presence of polymorphism (P/M) in 3% Agarose	Presence of polymorphism (P/M) in 6% PAGE
RM148	M	**P**	RM16	M	*
RM3867	M	M	RM570	M	*
RM6329	**P**	*	RM231	M	M
RM251	**P**	*	RM5626	**P**	*
RM293	M	M	RM554	M	*
RM218	M	*	RM563	M	*
RM60	M	M	RM3864	M	*
RM22	M	*	RM130	M	M
RM545	M	**P**	RM6283	M	*
RM7	**P**	*	RM411	M	*
RM282	M	M	RM36	**P**	*

*Not checked in Polyacrylamide gel electrophoresis
P- polymorphic markers between parents, M- Mormorphic markers between parents M- monomorphic

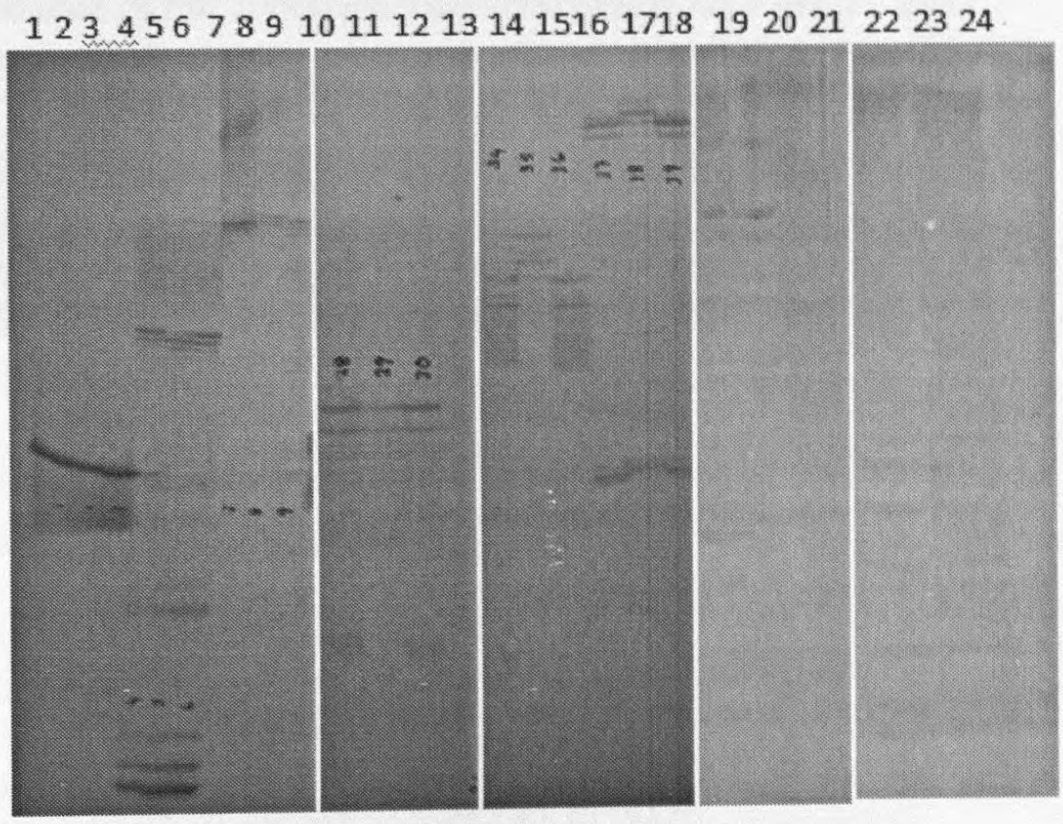


Figure 4.19. Polyacrylamide gel profile exhibiting monomorphic and polymorphic SSR markers in chromosome 3.

Lane1-3: RM 60, Lane 4-6: RM130, Lane 7-9, RM293, Lane 10-12:RM3867, Lane 13-15:RM148, Lane 16-18:RM545, Lane 19-21: RM282, Lane 22-24: RM231

Each marker represents three lanes amplified from DNA of At354 parent, Bg352 parent and RIL 4, respectively.

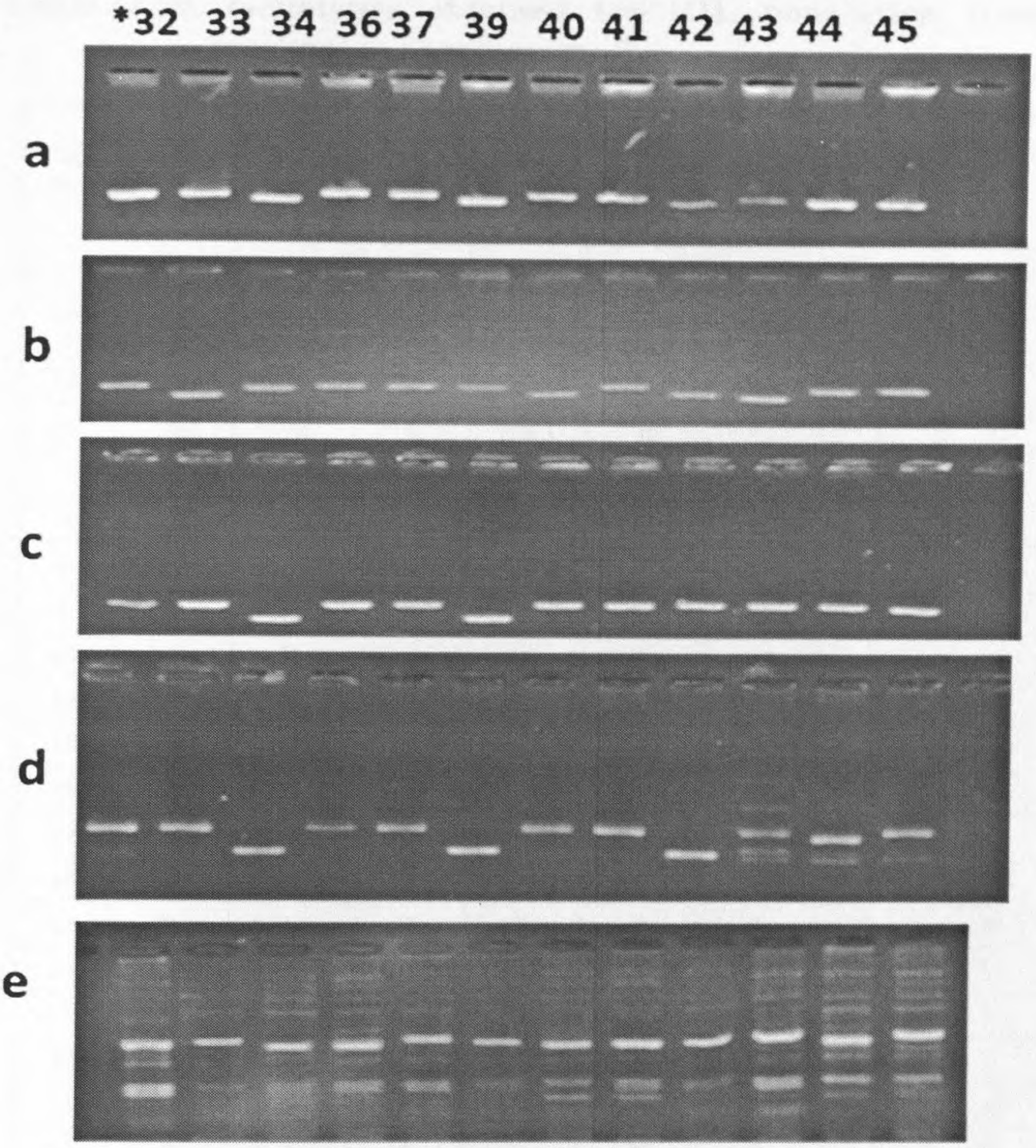


Figure 4.20. Polymorphism of some RILs amplified with markers in chromosome 3.

*Lanes are numbered based on RIL numbers.

a: RM7 (upper band: At352 genotype, lower band : Bg352 genotype)
b: RM5626 (upper band: At352 genotype, lower band : Bg352 genotype)
c: RM6329 (upper band: At352 genotype, lower band : Bg352 genotype)
d: RM251 (upper band: At352 genotype, lower band : Bg352 genotype)
e: RM36 (upper band: Bg352 genotype, lower band : At352 genotype)

Table 4.10. Genotypes obtained for RIL population from five polymorphic markers in chromosome 3.

Line No	RM7	RM5626	RM6329	Rm251	RM136	Line No	RM7	RM5626	RM6329	Rm251
1	A	A	A	A	A	56	A	A	A	A
2	A	A	A	A	A	58	A	B	A	A
3	A	A	B	A	B	59	A	B	A	A
4	A	A	A	A	B	61	A	A	A	A
5	A	B	A	A	A	64	A	B	A	A
7	A	A	A	A	B	66	A	A	A	A
8	A	A	A	A	A	71	A	A	H	A
9	A	A	A	A	A	72	A	A	A	A
12	A	B	A	A	A	74	A	A	A	A
14	A	A	A	A	A	76	A	A	A	A
15	B	B	B	B	A	77	A	B	A	A
16	A	A	A	A	A	78	A	B	A	A
17	A	A	A	A	A	80	A	A	A	A
18	A	A	A	A	A	82	B	A	H	B
19	B	A	B	B	B	83	A	B	A	A
20	A	A	A	A	A	84	A	B	A	A
21	A	A	B	A	A	85	A	A	A	A
22	A	A	A	A	A	87	A	B	A	A
23	B	B	B	B	A	89	A	A	A	A
24	A	A	A	A	B	90	A	B	A	A
25	A	A	A	A	A	95	A	A	A	A
29	A	A	A	A	A	96	A	B	A	A
30	A	A	A	A	A	97	B	B	B	B
31	A	A	A	A	B	98	A	B	A	A
32	A	A	A	A	A	99	A	h	A	A
33	A	B	A	A	B	100	A	B	A	A
34	B	A	B	B	A	102	A	B	A	A
36	A	A	A	A	A	103	A	A	A	A
37	A	A	A	A	B	105	A	B	A	A
39	B	A	B	B	A	106 A	A	A	A	A
40	A	B	A	A	A	106	A	A	A	A
41	A	A	A	A	A	107	A	A	A	A
42	B	B	A	B	A	109	A	B	A	A
43	A	B	A	A	B	110	A	A	A	A
44	A	A	A	A	A	111	A	A	A	A
45	A	A	A	A	B	113	A	A	A	A
47	A	B	B	A	A	114	A	A	A	A
50	A	B	A	A	A	117	B	B	H	B

51	A	A	A	A	B	**118**	A	A	A	A
53	A	A	A	A	B	**119**	A	h	A	A
121	A	A	A	A	A	**145**	A	A	A	A
123	B	B	B	B	B	**149**	A	A	B	A
124	B	A	A	-	A	**150**	A	A	A	A
125	A	A	A	A	A	**151**	A	A	A	A
134	A	A	A	A	A	**153**	A	B	A	A
137	A	A	A	A	A	**154**	A	A	A	A
138	A	B	A	A	A	**155**	A	B	A	A
145	A	A	A	A	A	**158**	A	A	A	A

A-At354 allele, B- Bg352 allele and h- heterozygous.

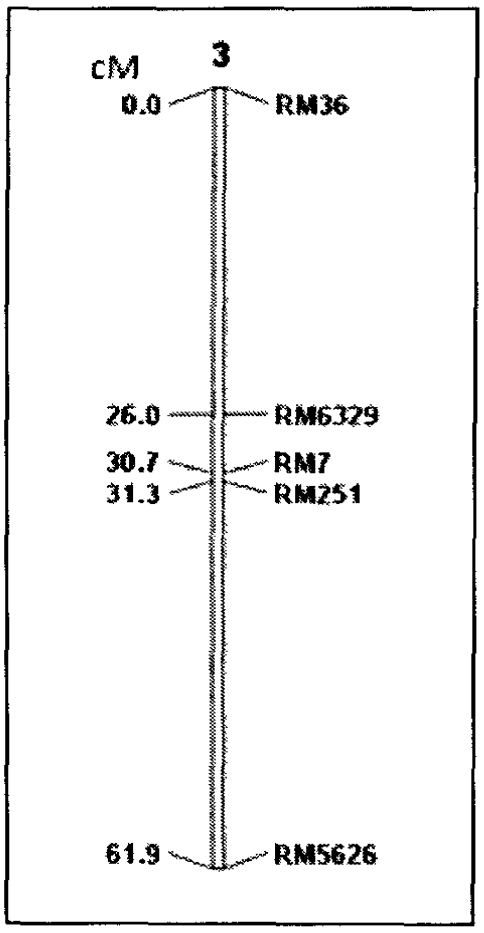


Figure 4.21. Linkage map of chromosome 3 resulted from 5 polymorphic SSR markers.
Distances are indicated in Kosambi centimorgans

Table 4.11. Probability values resulted from the single marker analysis (student's t-test) in chromosome 3.

Trait[a]	RM7	RM5626	RM6329	Rm251	RM36
RL	0.393 ^{ns}	0.665 ^{ns}	0.970 ^{ns}	0.301 ^{ns}	0.691 ^{ns}
SL	0.636 ^{ns}	0.437 ^{ns}	0.183 ^{ns}	0.659 ^{ns}	0.734 ^{ns}
RDW	0.549 ^{ns}	0.433 ^{ns}	0.078 ^{ns}	0.291 ^{ns}	0.466 ^{ns}
SDW	0.969 ^{ns}	0.267 ^{ns}	0.773 ^{ns}	0.867 ^{ns}	0.759 ^{ns}
SSI	0.783 ^{ns}	0.253 ^{ns}	0.234 ^{ns}	0.575 ^{ns}	0.152 ^{ns}
SNK	0.084 ^{ns}	0.726 ^{ns}	0.418 ^{ns}	0.128 ^{ns}	0.169 ^{ns}
SNC	0.089 ^{ns}	0.471 ^{ns}	0.287 ^{ns}	0.124 ^{ns}	0.219 ^{ns}
SKC	0.147 ^{ns}	0.229 ^{ns}	0.042 ^{ns}	0.207 ^{ns}	0.178 ^{ns}

[a]Root length(RL), Shoot length (SL), Root dry weight (RDW), Shoot dry weight (SDW), Salinity Survival Index (SSI), Shoot Na^+ concentration (SNC), Shoot K^+ concentration (SKC) and Shoot Na^+/K^+ (SNK) ratio,

^{ns}*Not significant*

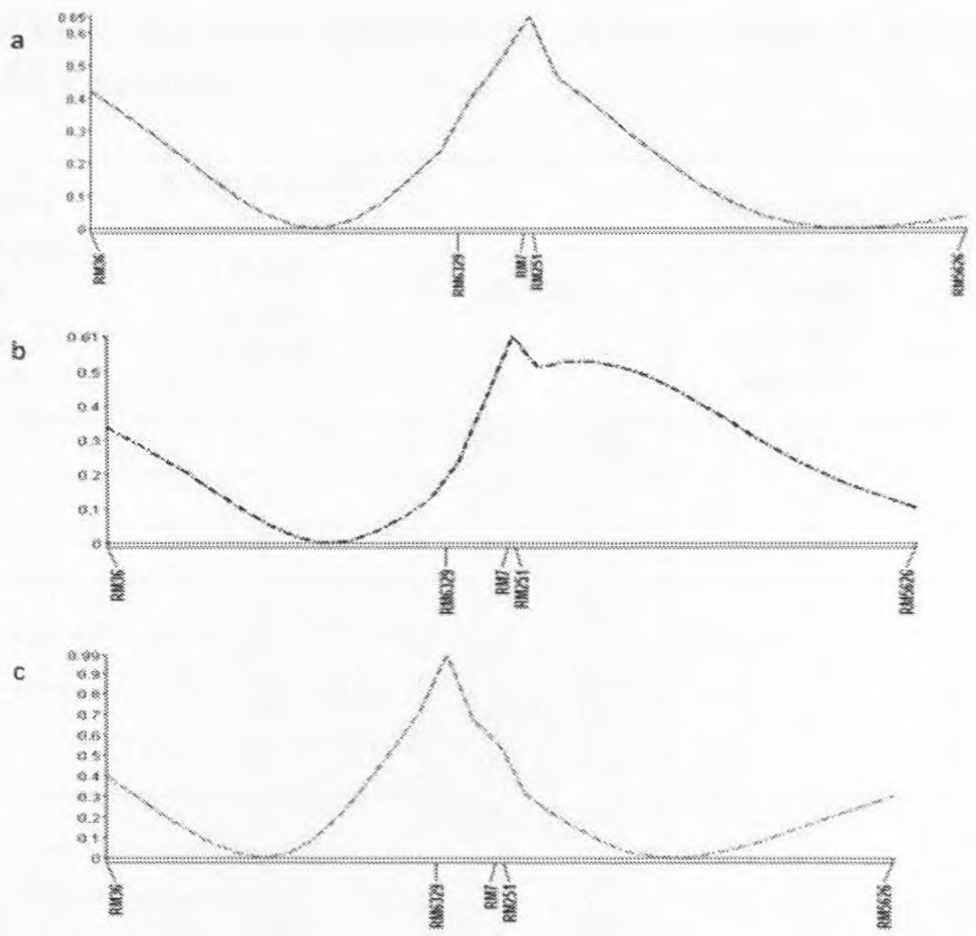


Figure 4.22. Few QTL maps showing low LOD values in chromosome 3.

(a) Shoot Na^+/K^+ .
(b) Shoot Na^+ concentration.
(c) Shoot K^+ concentration.

4.13. Polymorphism between parental genotypes and genotyping with polymorphic markers in chromosome 4.

Nine polymorphic markers from twenty six were found polymorphic in chromosome 4 using 3% agarose (Table 4.12) (Figure 4.23 a and b). In 6 % Polyacrylamide, only one marker showed polymorphism for parental germplasm, out of five markers which were monomorphic in 3% agarose gel electrophoresis (Figure 4.24). The percentage of polymorphic markers was 38.4% in chromosome 4 (Table 4.22). Genotypic data obtained from the amplification of RILs with polymorphic markers are shown in figure 4.25 a and b and table 4.13.

4.14. Construction of linkage map of chromosome 4.

The linkage map of chromosome 4 constructed from the genotypic data obtained from polymorphic markers contained only 5 markers as other markers were not linked with each other (Figure 4.26). The 5 markers were distributed throughout the genome with 19.38 cM average. The distance between first two markers R4M17 and RM335 was 14.2 cM. The linkage distance between RM335-RM518 and RM518-RM5749 marker pairs were 9.1 cM and 26.9 cM respectively. The maximum distance between markers observed was 46.9 cM for RM5749 and RM280.

Table 4.12. Screening of SSR markers located on chromosome 4 for polymorphism between At354 and Bg352 parents

Primer	Presence of polymophism (P/M) in 3% Agarose	Presence of polymophism (P/M) in 6% PAGE	Primer	Presence of polymophism (P/M) in 3% Agarose	Presence of polymophism (P/M) in 6% PAGE
R4M17	**P**	*	RM335	**P**	*
R4M43	M	*	RM261	M	M
R4M50	M	*	RM470	M	*
R4M13	M	*	RM280	**P**	*
RM127	M	*	RM142	M	*
RM273	**P**	*	RM119	M	*
RM518	**P**	*	RM559	**P**	*
RM307	M	*	RM1272	M	*
RM3843	**P**	*	RM6303	M	**P**
RM241	M	M	RM17708	M	*
RM5749	**P**	*	RM17693	**P**	*
RM317	M	*	RM1113	M	M
RM2636	M	*	RM3335	M	M

*Not check in Polyacrylamide gel electrophoresis

P- polymorphic markers between parents, M- Mormorphic markers between parents

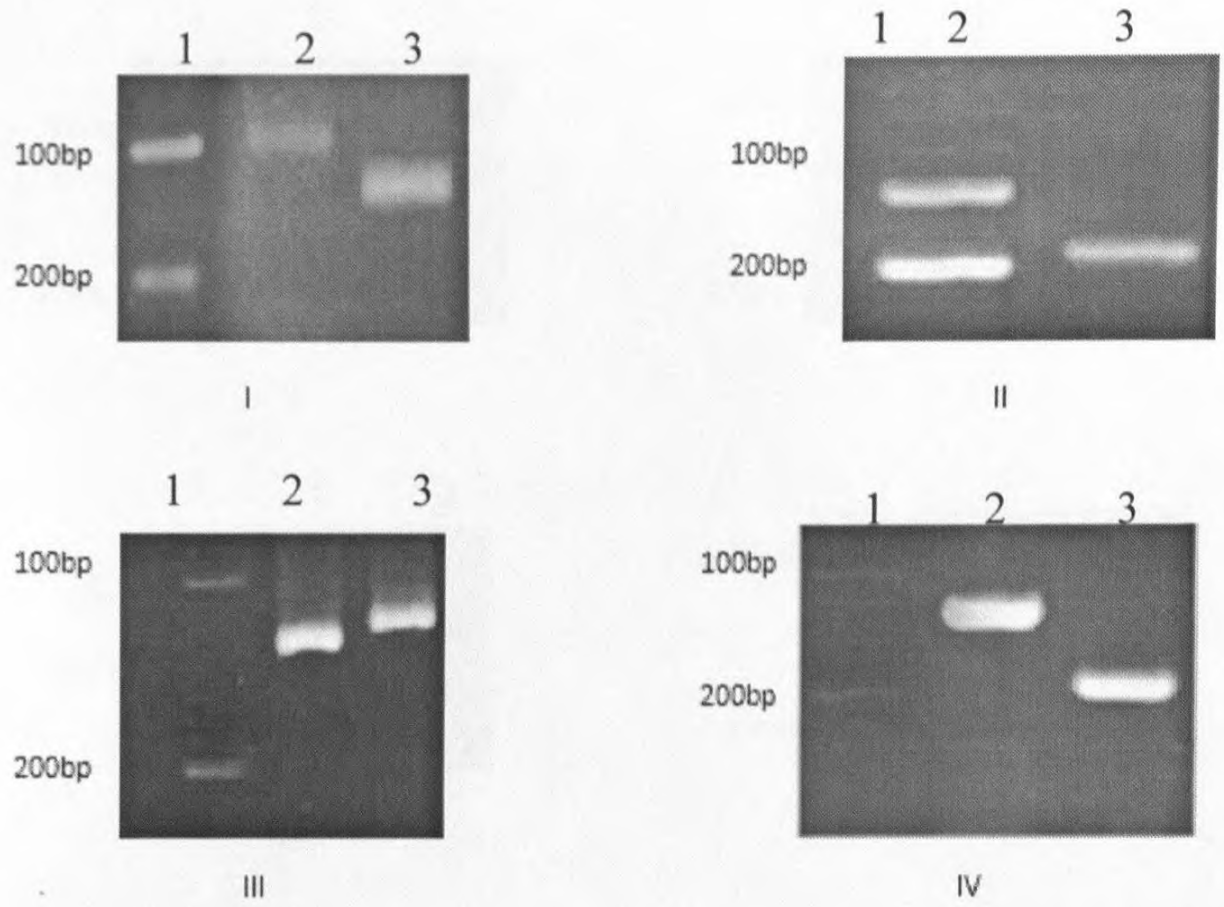


Figure 4.23a. PCR amplification with R4M17, RM335, RM518 and RM5749 SSR markers located on chromosome 4 showing polymorphism between At354 and Bg352 parental lines

I . R4M17 II . RM335 III . RM518 IV. RM5749

Lane 1- 100bp DNA ladder, Lane 2- At354, Lane 3- Bg352

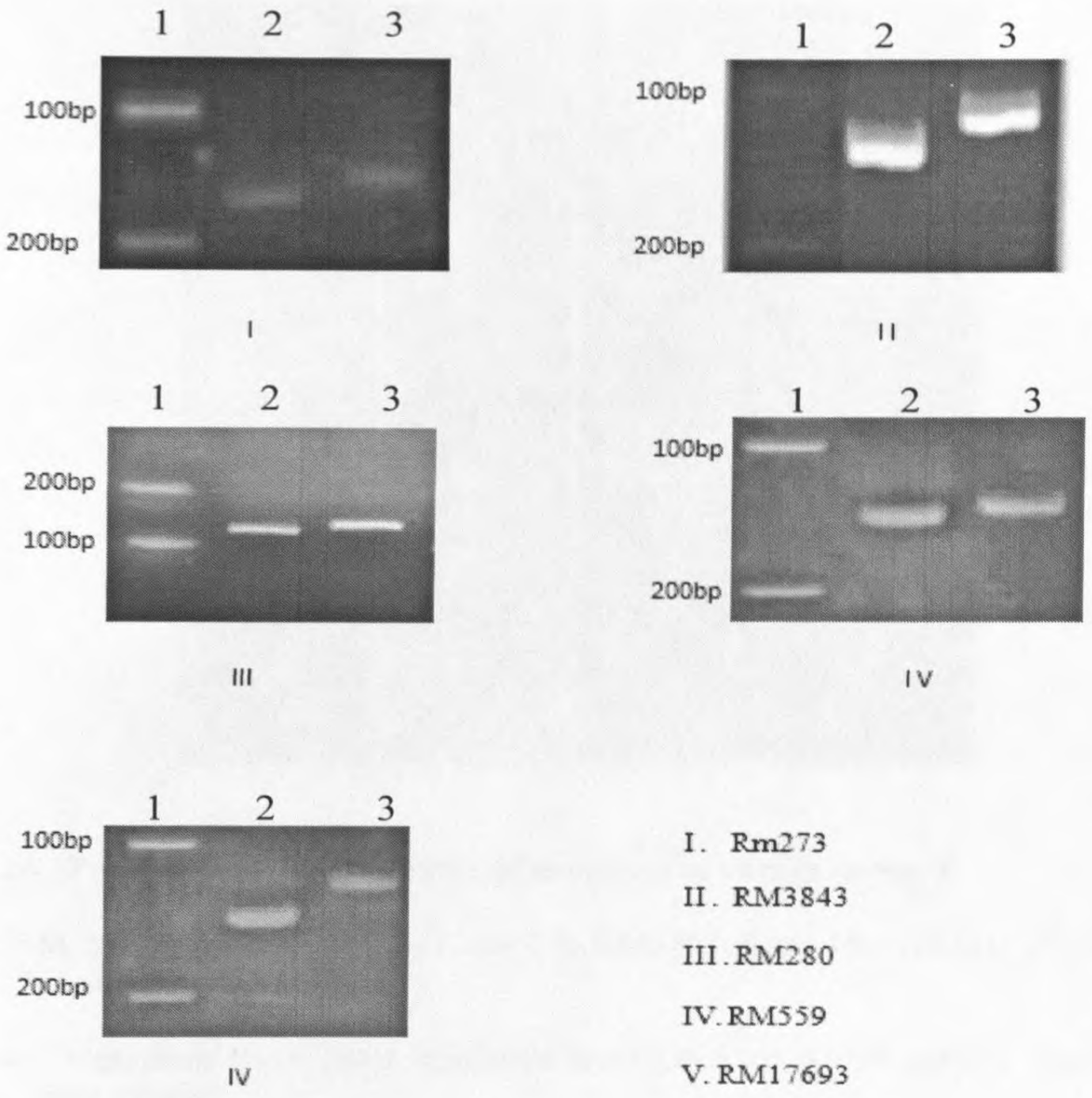


Figure 4.23b. PCR amplification with RM273, RM3843, RM280, RM559 and SSR markers located on chromosome 4 showing polymorphism between At354 and Bg352 parental lines

Lane 1- 100bp DNA ladder, Lane 2- At354, Lane 3- Bg352

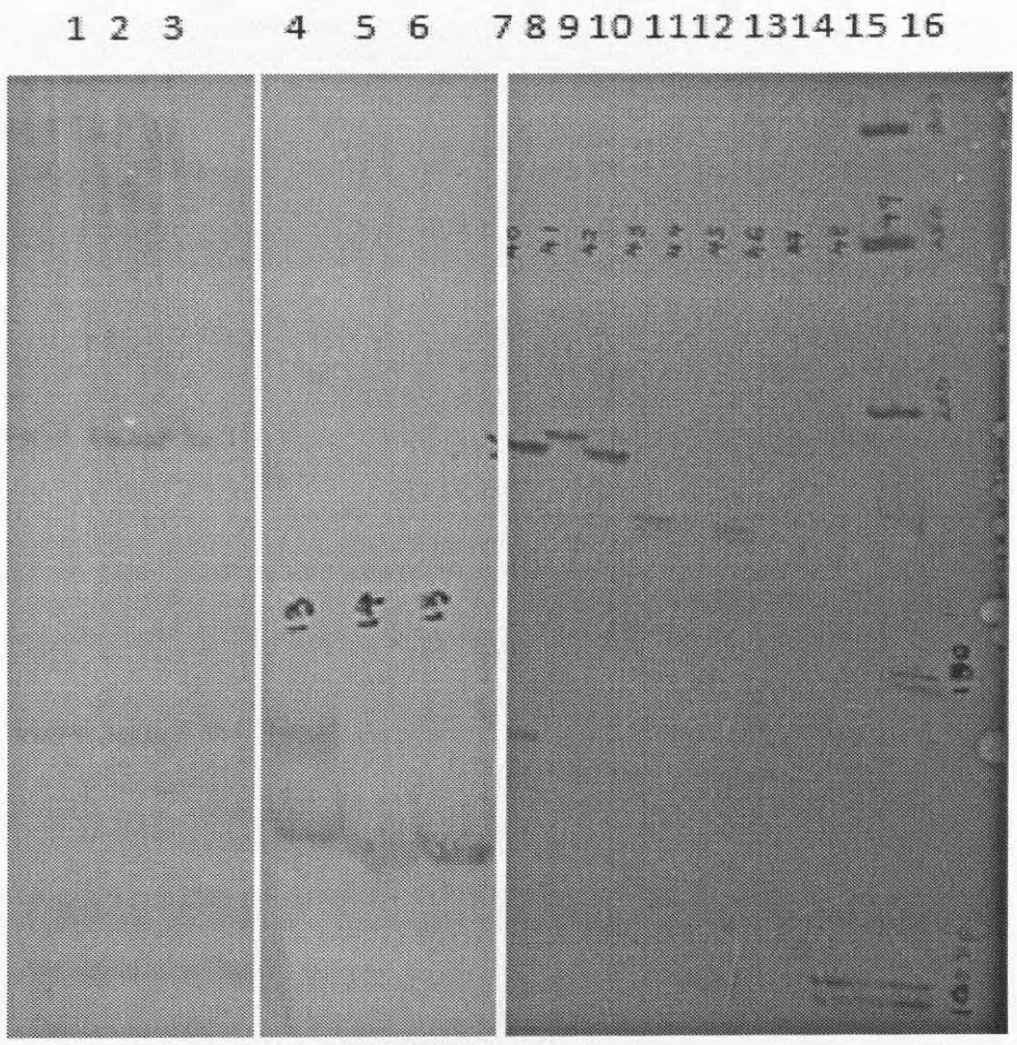


Figure 4.24. Polyacrylamide gel profile of markers in chromosome 4.

Lane 1-3: RM 241, Lane 4-6: RM261, Lane 7-9, RM6303, Lane 10-12:RM1113, Lane 13-15:RM3335, Lane 16:100bp ladder

Each marker represents three lanes amplified from DNA of At354 parent, Bg352 parent and RIL No. 4, respectively

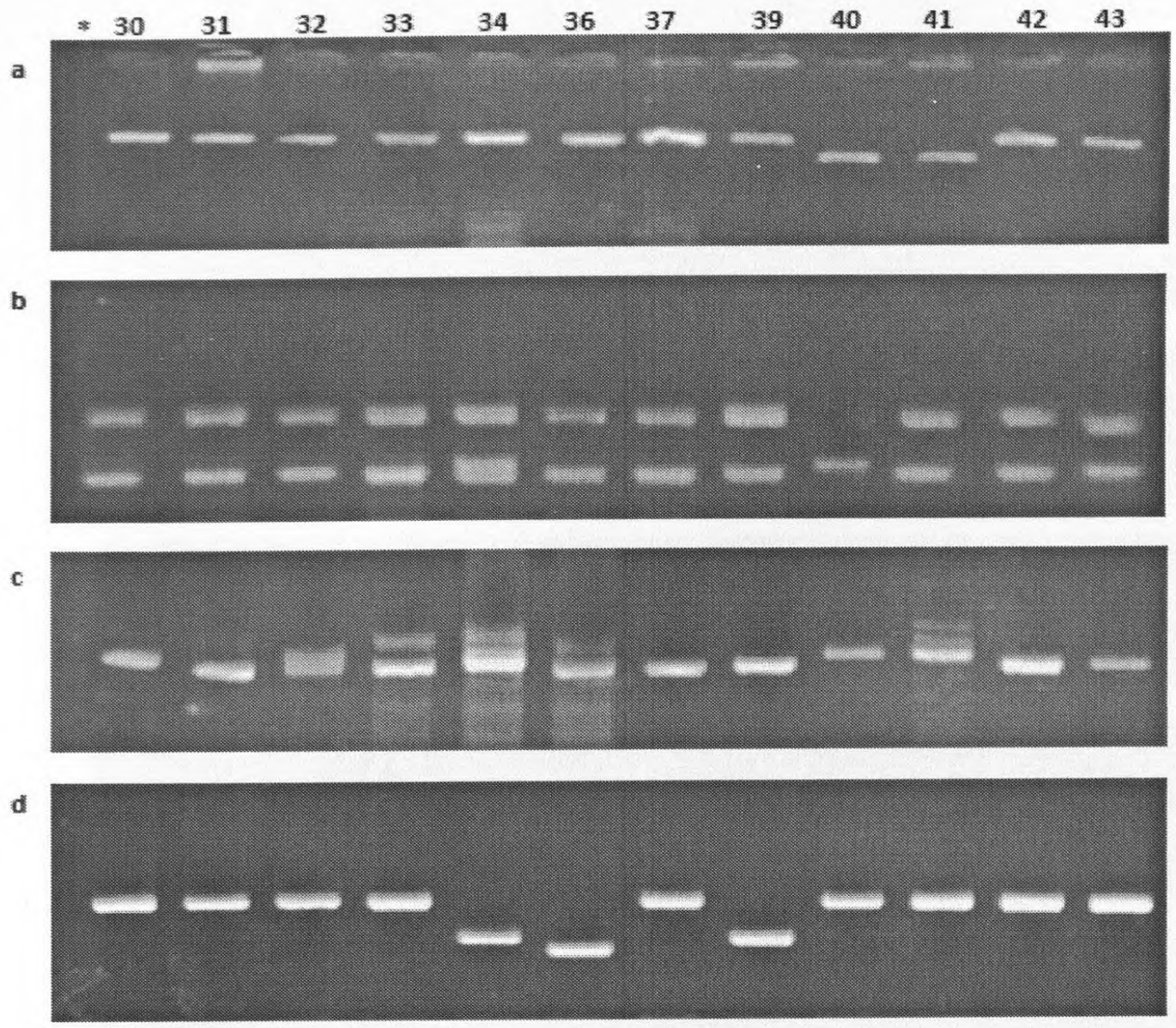


Figure 4.25a. Genotyping profile of some RILs amplified with R4M17, RM335, RM518 and RM5749 markers in chromosome 4

**Lanes are numbered based on RIL numbers.*

a: R4M17 (upper band: At352 genotype, lower band : Bg352 genotype)
b: RM 335 (double bands: At352 genotype, single band : Bg352 genotype)
c: RM 518 (upper band: Bg352 genotype, lower band : At354 genotype)
d: RM 5749 (upper band: At352 genotype, lower band : Bg352 genotype)

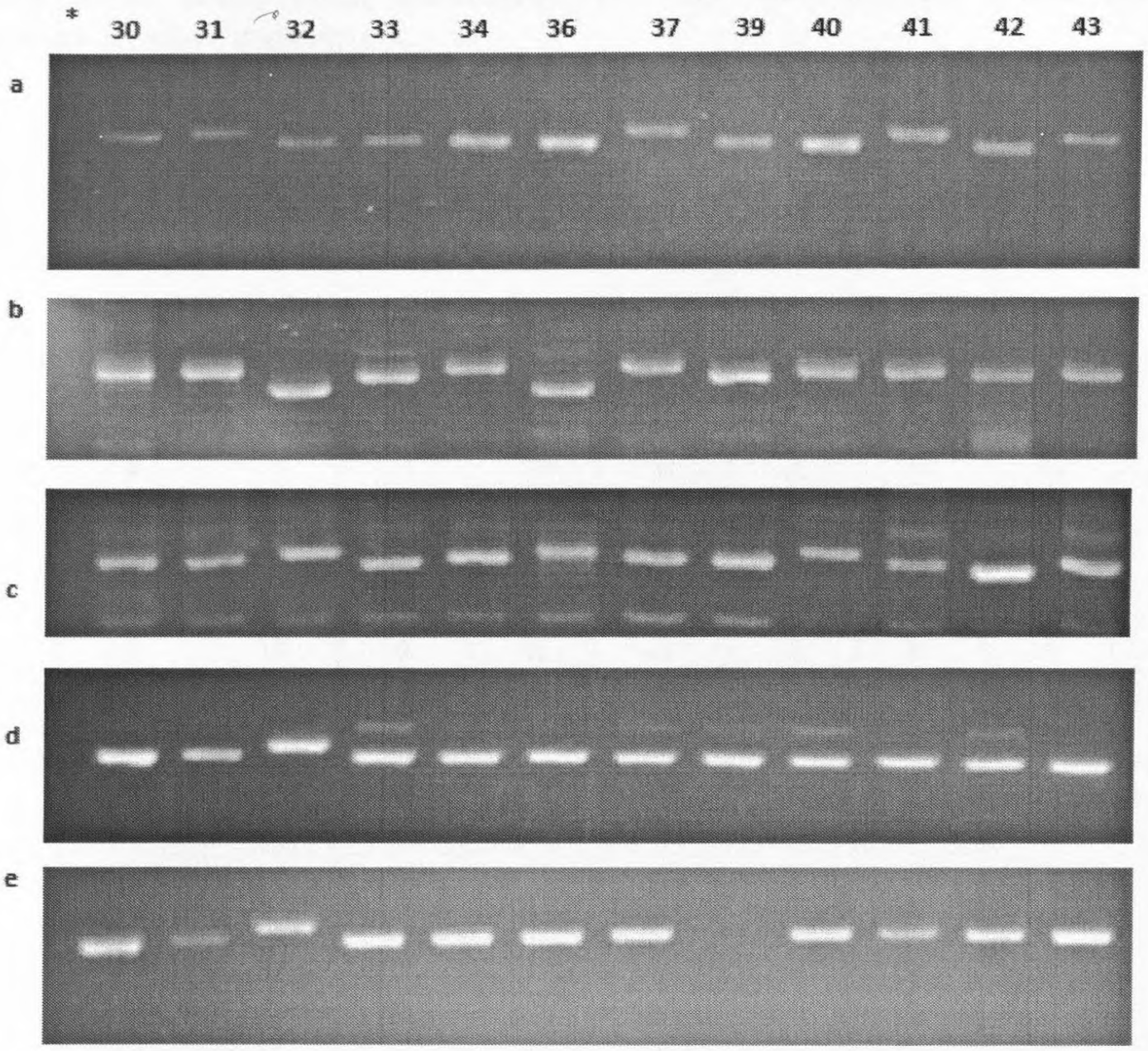


Figure 4.25b. Genotyping profile **of some RILs amplified with RM273, RM3843, RM280, RM559 and RM17693 SSR markers in chromosome 4**

**Lanes are numbered based on RIL numbers.*

a: R273 (upper band: Bg352 genotype, lower band : At352 genotype)
b: RM 3843 (upper band: Bg352 genotype, lower band : At352 genotype)
c: RM 280 (upper band: Bg352 genotype, lower band : At354 genotype)
d: RM 559 (upper band: Bg352 genotype, lower band : At352 genotype)
e: RM 17693 (upper band: Bg352 genotype, lower band : At352 genotype)

Table 4.13. Genotypes obtained for RIL population from nine polymorphic markers in chromosome 4.

	R_4M_{17}	RM335	RM518	RM5749	RM273	RM3843	RM280	RM559	RM17693
1	B	A	A	A	A	B	B	A	A
2	A	A	A	A	B	B	B	A	A
3	A	A	A	A	A	B	B	A	A
4	A	A	A	B	A	B	B	A	A
5	A	A	A	A	B	B	B	A	A
7	A	A	A	B	A	B	B	A	A
8	A	A	A	A	A	B	A	A	A
9	B	A	A	A	A	B	A	A	A
12	A	A	B	A	B	B	B	A	A
14	A	A	A	A	A	B	A	A	A
15	B	B	B	A	A	A	B	A	A
16	A	A	A	A	A	B	A	A	A
17	A	A	A	A	B	B	B	A	A
18	H	A	A	A	A	B	B	A	A
19	A	A	A	A	A	A	B	A	A
20	A	A	A	A	A	B	B	A	A
21	B	A	A	A	A	A	B	A	A
22	B	A	A	A	A	B	A	A	A
23	A	B	A	B	A	A	B	A	A
24	A	A	A	A	A	B	B	A	A
25	B	A	A	A	A	B	B	A	A
29	A	A	A	A	A	B	A	A	A
30	A	A	A	A	B	B	A	A	A
31	A	A	A	A	B	B	A	A	A
32	A	A	B	A	A	B	A	A	A
33	A	A	A	A	B	B	A	A	A
34	A	A	A	A	A	A	B	B	B
36	A	A	A	A	A	B	A	A	A
37	A	A	A	B	A	B	B	A	A
39	A	A	A	B	A	A	B	A	A
40	B	A	A	A	B	B	A	A	A
41	B	A	A	B	A	B	A	A	A
42	A	B	B	A	A	B	B	A	A
43	A	A	B	A	B	B	A	A	A
44	A	A	A	A	A	B	A	A	A
45	A	A	A	A	B	B	A	A	A
47	B	B	B	A	-	A	B	A	A
50	A	A	B	B	B	B	B	A	A
51	A	A	A	A	A	A	B	A	A
53	A	A	B	B	A	B	B	A	A

54	H	A	A	A	A	B	B	A	A
56	A	A	A	B	A	B	B	A	A
58	A	A	B	B	A	B	B	A	A
59	A	A	A	B	A	B	B	A	A
61	A	A	A	A	B	B	B	A	A
64	A	A	A	A	B	B	A	A	A
66	A	A	B	A	B	B	A	A	A
71	A	A	A	A	B	B	A	A	A
72	A	A	A	A	A	A	A	A	A
74	B	A	A	A	A	B	B	A	A
76	A	A	A	A	B	B	A	A	A
77	A	A	A	B	A	A	A	A	A
78	A	A	A	B	B	B	A	A	A
80	A	A	A	A	A	A	A	A	A
82	A	A	A	h	A	B	B	B	B
83	A	A	B	B	A	A	A	A	A
84	A	A	A	A	A	A	A	A	A
85	A	A	A	A	A	A	A	A	A
87	A	A	A	A	A	B	A	A	A
89	A	A	A	B	A	B	-	A	A
90	A	A	A	A	A	A	A	A	A
95	B	A	A	A	A	B	A	A	A
96	A	A	A	A	B	A	B	A	A
97	A	A	A	A	A	A	B	B	B
98	A	A	A	B	A	B	A	A	A
99	A	A	A	B	B	B	A	A	A
100	A	A	B	h	A	B	A	A	A
102	A	A	A	A	B	B	A	A	A
103	H	A	A	A	A	B	A	A	A
105	A	A	A	B	A	B	B	A	A
106 A	A	A	A	A	A	B	B	A	A
106	A	A	A	B	B	B	B	A	A
107	A	A	A	A	A	A	A	A	A
109	A	A	A	A	B	A	A	A	A
110	B	A	A	A	A	A	B	A	A
111	B	A	A	A	A	A	A	A	A
113	A	A	A	A	A	B	-	A	A
114	B	A	A	A	A	A	A	A	A
117	A	A	A	A	A	A	A	A	A
118	A	A	A	A	A	A	h	A	A
119	A	A	A	A	A	B	A	A	A
121	A	A	A	A	A	B	A	A	A
123	B	B	A	B	A	B	A	B	B
124	-	A	A	A	A	B	A	A	A
125	A	A	B	A	A	B	A	A	A
134	A	A	B	A	A	A	A	A	A
137	A	A	A	A	A	B	A	A	A

138	A	A	A	A	A	B	A	A	A
145	B	A	A	A	A	A	A	A	A
149	A	A	A	A	A	A	A	A	A
150	A	A	A	A	A	A	A	A	A
151	B	A	A	A	A	B	A	A	A
153	A	A	A	B	A	A	A	A	A
154	A	A	A	B	B	B	A	A	A
155	A	A	A	A	B	B	A	A	A
158	B	A	A	A	A	B	B	h	H

- At354 allele, B- Bg352 allele and h- heterozygote.

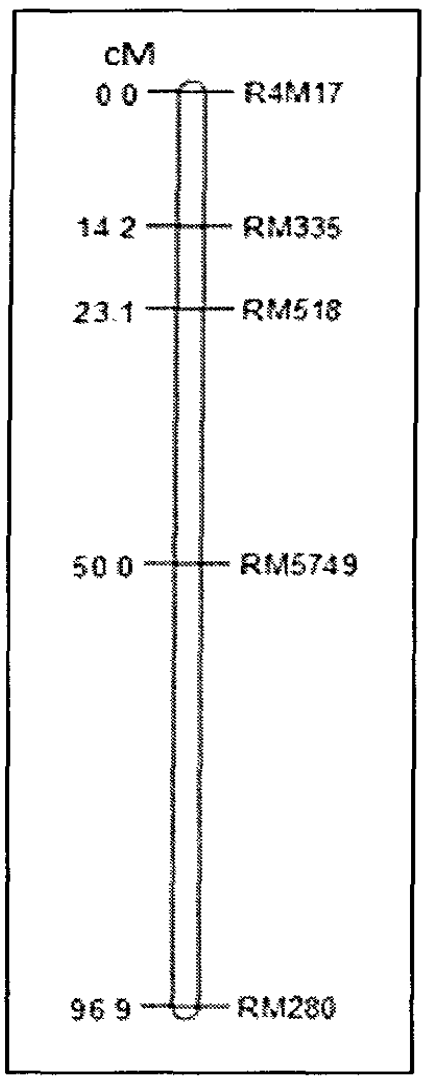


Figure 4.26. Linkage map of chromosome 4 resulted from 5 polymorphic SSR markers. Distances are indicated in Kosambi centimorgans

4.15. Detection of QTLs in chromosome 4

4.15.1. Detection of QTLs by single marker analysis method

Student t- test results, conducted to detect the relationship between quantitative traits showed that 5 markers in chromosome 4 were significantly associated with five quantitative characters measured, SSI, shoot length and shoot Na+/K+, shoot Na+ level and shoot K+ level (Table 4.14) indicating existence of putative QLTs for those characters.

4.15.2. Mapping of QTLs by single marker regression

Single marker regression results showed the presence of two putative QTLs with LOD 2 at RM280 (*qSSI4*) and LOD 3 at RM335 (*qSNK4*) for SSI and shoot Na+/K+ traits respectively. According to the results obtained from 1000 permutation test, QTL for SSI was significant at 0.05 and QTL for shoot Na+/K+ was significant at 0.01 level. It could be explained 9.2% phenotypic variation in SSI by the QTL *qSSI4* and 14.1% phenotypic variation in shoot Na+/K+ by *qSNK4* (Table 4.15 and figure 4.27).

4.15.3 Mapping of QTLs by composite interval mapping

Three QTLs in chromosome 4 were detected from the composite interval mapping responsible for the phenotypic variation in shoot length, Na^+/K^+ and SSI (Figure 4.28-4.30). QTL identified for shoot length was significant at 0.05 level with 2.4 LOD, which located in RM3843-RM280 and it could explain eleven percent of the variation exhibited in the mapping population. According to the Table 4.15 and Figure 4.29, At354 allele contributed to the increment of shoot length by 3.3 units. The position of the QTL identified for shoot Na^+/K^+ by SMR has been shifted towards RM518 in CIM. In this QTL At354 allele has contributed to decrease Na^+/K^+ by 2.25 units (Table 4.15 and Figure 4.30). This QTL could elucidate 16 % of the Na/K variation in RIL population with 3.6 LOD score, which was significant at 0.01 level. The QTL responsible for SSI was significant at 0.01 level and it was located between RM3843-RM280 markers with 3.3 LOD score (Table 4.15). The contribution of the QTL to the variation of SSI among RILs was 15% and At354 allele contributed to increase the SSI by 0.14 units as appeared in additive effect graph (Figure 4.28 and Table 4.15).

Table 4.14. Probability values resulted from the single marker analysis (student's t-test) in chromosome 4

Trait[a]	R4M17	RM335	RM518	RM5749	RM3843	RM280	RM273	RM559	RM17693
RL	0.259	0.567	0.580	0.485	0.078	0.242	0.617	0.649	0.649
SL	0.732	0.898	0.511	0.916	**0.033***	**0.016***	0.691	0.662	0.663
RDW	0.154	0.333	0.585	0.137	0.519	0.051	0.561	0.938	0.939
SDW	0.421	0.407	0.917	0.283	0.7417	0.942	0.518	0.799	0.799
SSI	0.722	0.310	0.729	0.385	**0.024***	**0.004****	0.504	0.369	0.369
SNK	0.205	0.834	**0.0003*****	0.070	0.280	0.191	0.094	**0.009****	**0.009****
SNC	0.272	0.655	**0.008****	0.363	0.142	0.112	0.328	**0.005****	**0.005****
SKC	0.298	0.505	**0.0161***	0.179	0.689	0.775	0.050	0.229	0.229

[a]Root length(RL), Shoot length (SL), Root dry weight (RDW), Shoot dry weight (SDW), Salinty Survival Index (SSI), Shoot Na^+ concentration (SNC), Shoot K^+ concentration (SKC) and Shoot Na^+/K^+ (SNK) ratio

**Significant at 0.05, **Significant at 0.01, ***Significant at 0.001, [ns]Not significant*

Table 4.15. Identified putative QTLs in chromosome 4 for 3 morpho-physiological traits assessed in RIL population derived from At354/Bg352 cross under salinity stress

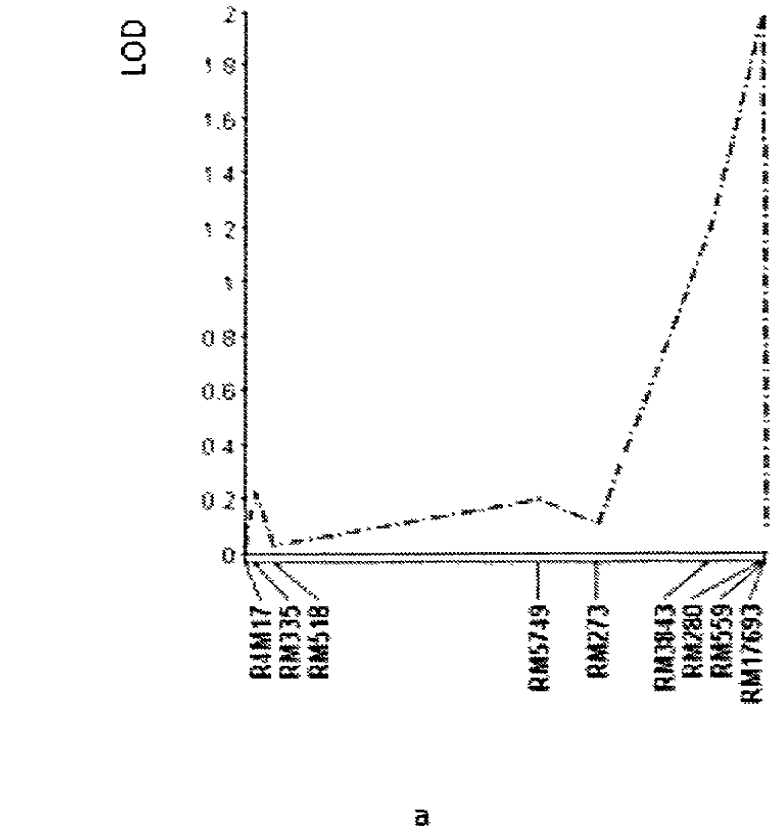
QTL analysis method[a]	Trait[b]	QTL	Chr	Peak marker	Flanking markers		Peak Positio -n (cM)	Peak Positio -n (Mb)	Add. effect[c]	LOD	R^2 (%)	LOD threshold		Salt toleran t allele Donor[d]
												α = 0.05	α = 0.01	
SMR	SSI	*qSSI4*	4	RM280	RM3843	RM559	139.96	34.99	0.068	2	14.1	1.63	2.078	At354
	SNK	*qSNK4*	4	RM335	R4M17	RM518	4.75	0.688	-0.64	3	9.2	1.686	2.453	At354
CIM	SSI	*qSSI4*	4	RM280	RM3843	RM280	139.96	34.99	0.14	3.3	15	1.834	2.461	At354
	SL	*qSL4*	4	RM280	RM3843	RM280	139.96	34.99	3.3	2.4	11	1.812	2.624	At354
	SNK	*qSNK4*	4	RM518	RM518	RM5749	8.12	2.03	-2.25	3.6	16	1.775	2.458	At354

[a] SMR - Single marker regression, CMI- Composite interval mapping

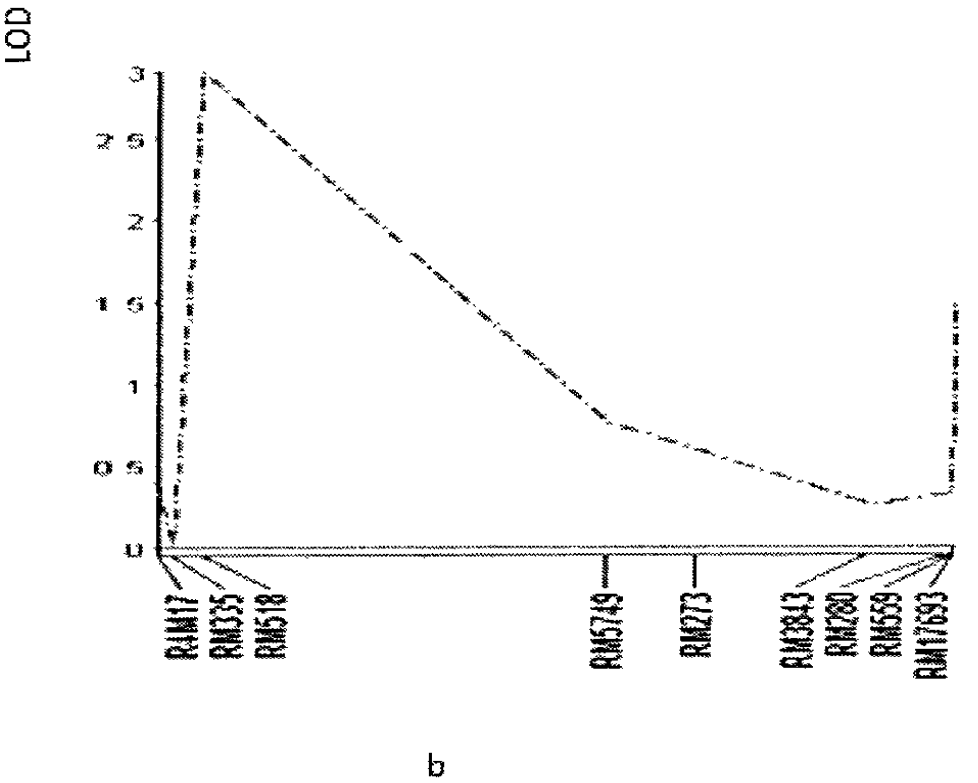
[b] Salinity survival index (SSI), Shoot length (SL), Shoot Na+ concentration (SNC), Shoot K+ concentration(SKC) and Shoot Na+/K+(SNK) ratio
Chr - chrchromosome

[c] positive value means At354 alleles contribute to increase effect of respective trait, negative value means Bg352 alleles contribute to increase effect of respective trait

[d] Allele donor in favour of salt tolerance



a



b

Figure 4.27. QTL maps of salinity survival index and shoot Na^+/K^+ ratio on chromosome 4 resulted by single marker regression (SMR).

a- QTL map of salinity survival index b- QTL map of shoot Na^+/K^+ ratio

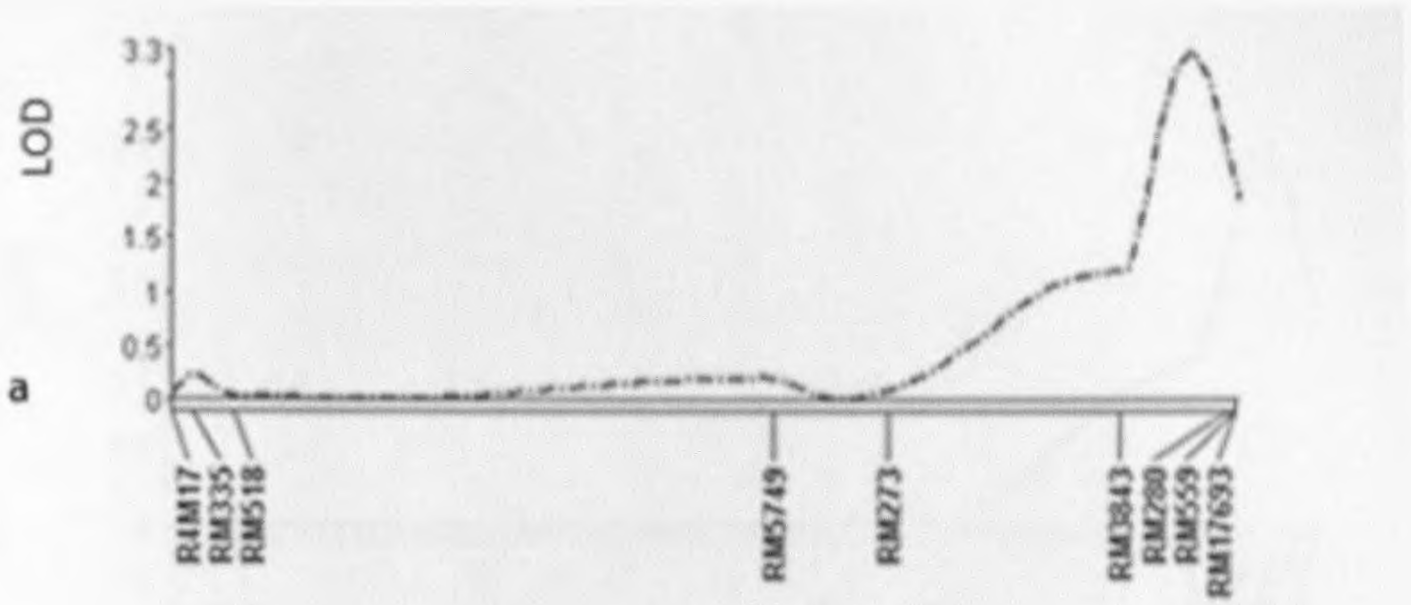


Figure 4.28. QTL map and additive effect graph of salinity survival index on chromosome 4 resulted by composite interval mapping (CIM)

a- QTL map of salinity survival index showing the location of *qSSI4*
b- Graph of additive effect indicating contribution of At354 and Bg352 alleles for salinity survival

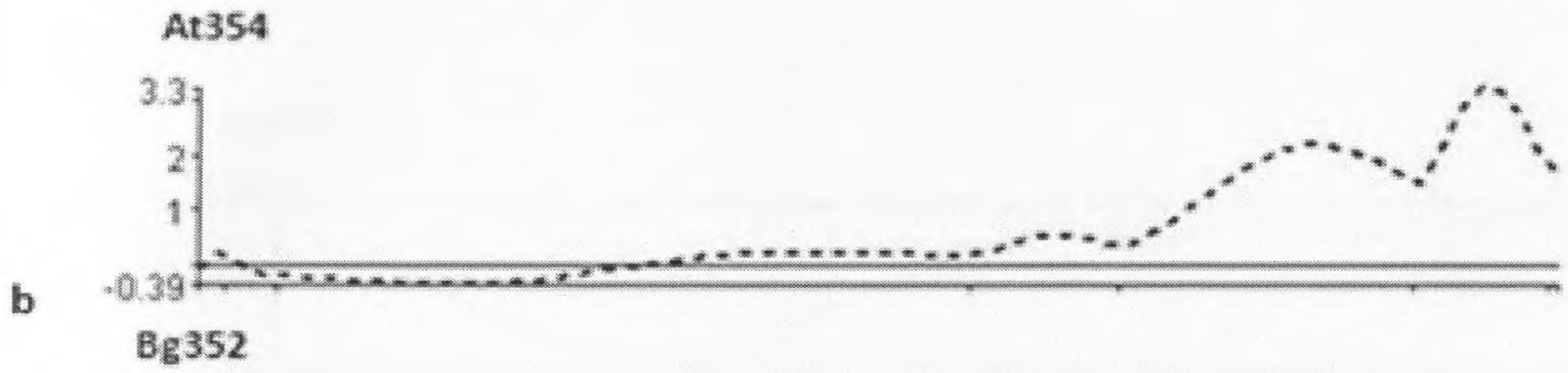


Figure 4.29. QTL map and additive effect graph of shoot length on chromosome 4 resulted by composite interval mapping (CIM)

a- QTL map of shoot length showing the location of *qSL4*
b- Graph of additive effect indicating contribution of At354 and Bg352 alleles for salinity survival

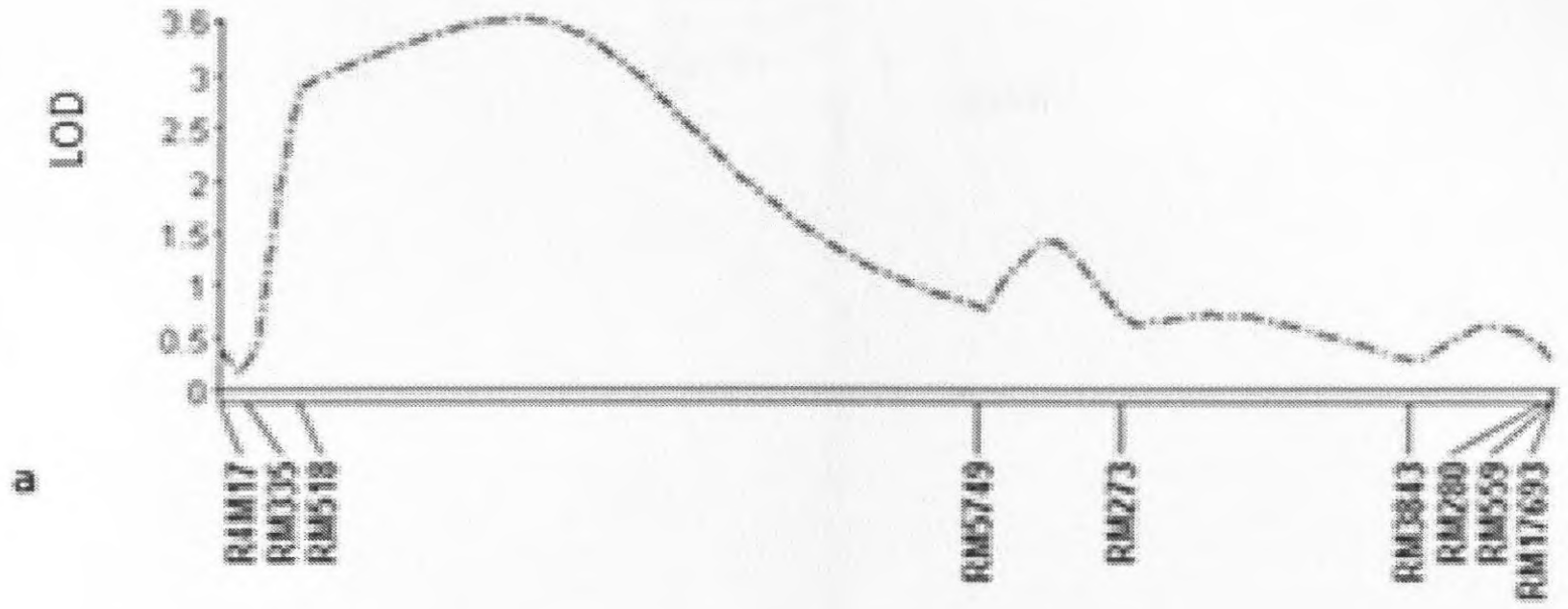


Figure 4.30. QTL map and additive effect graph of shoot Na^+/K^+ ratio on chromosome 4 resulted by composite interval mapping (CIM)

a- QTL map of shoot Na^+/K^+ ratio showing the location of *qSNK4*
b- Graph of additive effect indicating contribution of At354 and Bg352 alleles for salinity survival

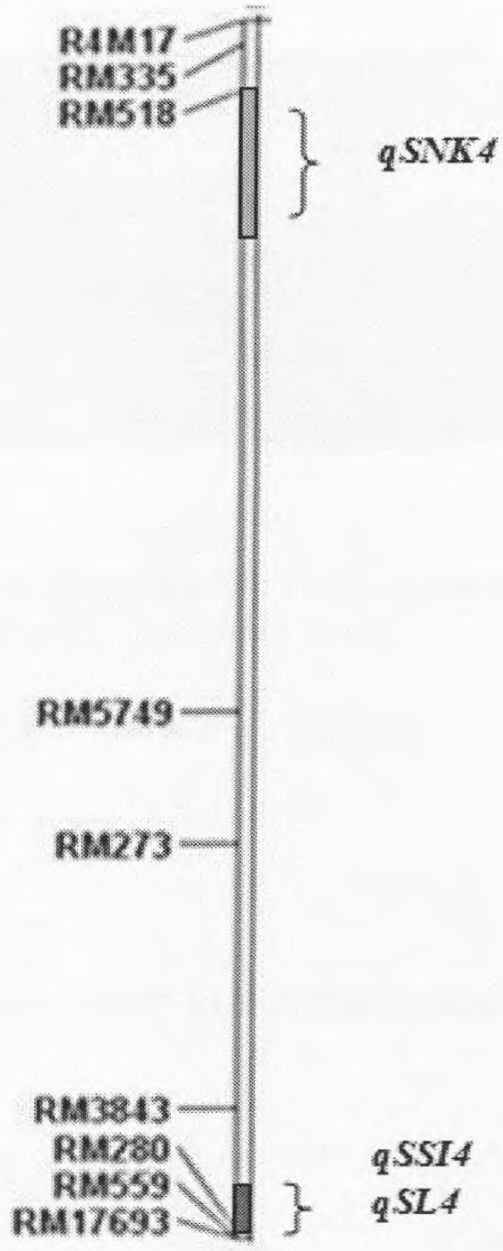


Figure 4.31. Putative QTLs identified on chromosome 4 using composite interval mapping (CIM).

qSSI4 – QTL identified for salinity survival index
qSL4- QTL identified for shoot length
qSNK4 - QTL identified for shoot Na+/K+ ratio

4.16. Polymorphism in parents with molecular markers in chromosome 12 and genotyping

For chromosome 12, out of 13 markers analyzed only RM1261 showed polymorphism in 3 % agarose (Figure 4.32). One marker RM511, out of 8 markers which were not polymorphic in agarose was found to be polymorphic in 6% PAGE (Table 4.16) (Figure 4.33). The total polymorphism percentage observed for chromosome 12 was 26% (Table 4.22). RM 1261 was used to genotype the RILs and there were 54 individuals showing At354 allele and 39 were showing Bg352 allele. Results obtained from student t test showed that there is no association between the At345 and Bg352 genotypes for any of the traits measured at RM1261 (Table 4.17).

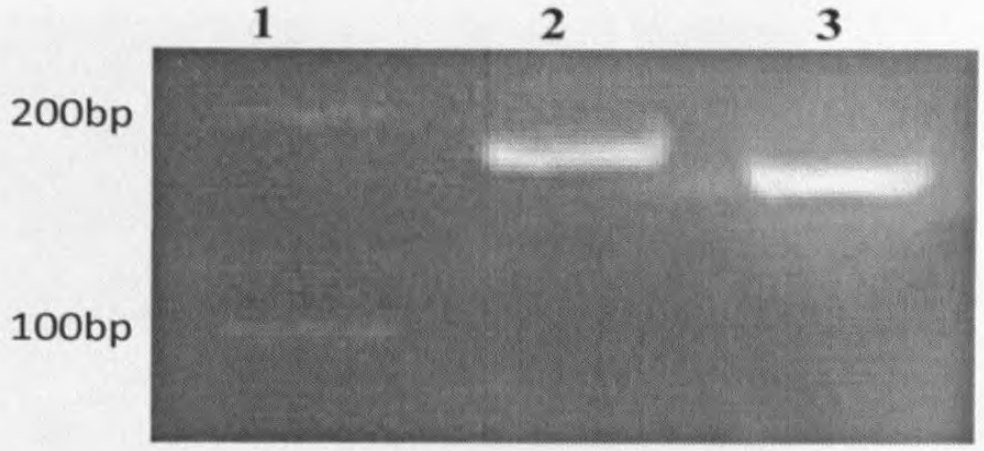


Figure 4.32. PCR amplification with RM1261 located on chromosome 12 showing polymorphism between At354 and Bg352 parental lines.

Lane 1- 100bp DNA ladder, Lane 2- At354, Lane 3- Bg352

Table 4.16. Surveyed molecular markers in chromosome 12 and their polymorphic status

primer	Presence of polymophism (P/M) in 3% Agarose	Presence of polymophism (P/M) in 6% PAGE	Primer	Presence of polymophism (P/M) in 3% Agarose	Presence of polymophism (P/M) in 6% PAGE
R12M27	M	*	RM270	M	M
RM102	M	*	RM511	M	**P**
RM28102	M	M	Ba76H14	M	*
RM1261	**P**	*	RM277	M	M
RM519	M	M	RM3326	M	M
RM247	M	M	RM491	M	*
RM463	M	M			

*Not checked in Polyacrylamide gel electrophoresis

P- polymorphic markers between parents, M- Monomorphic markers between parents

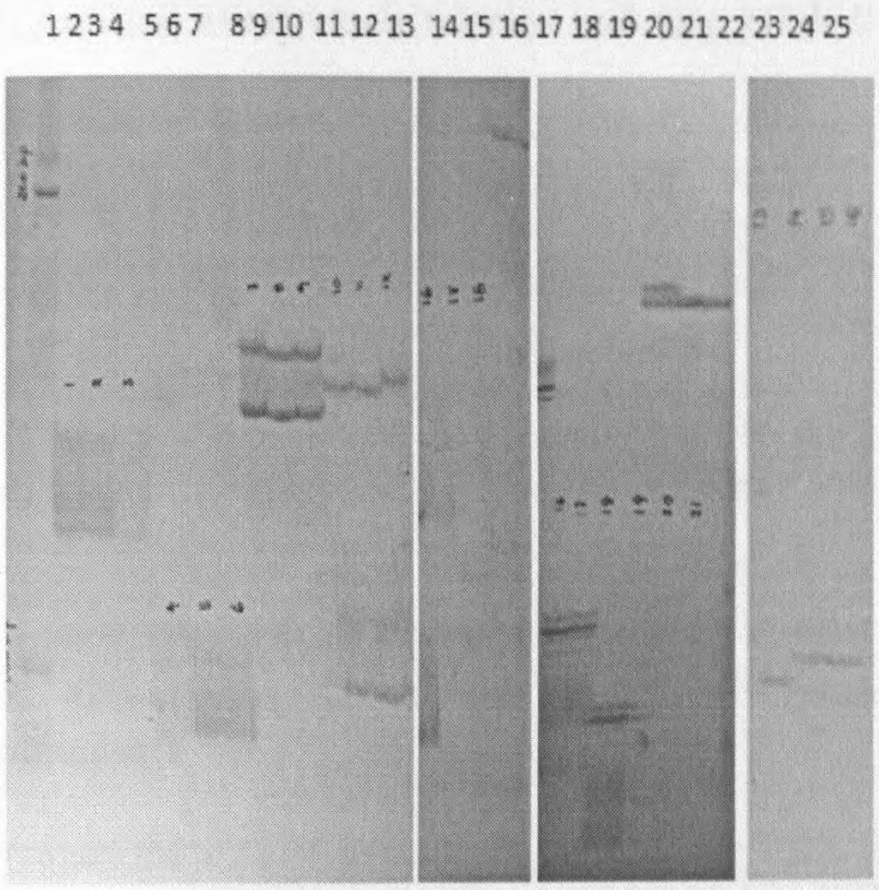


Figure 4.33 Polyacrylamide gel profile of chromosome 12 markers.

Lane1: 100bp DNA Ladder, Lane 2-4: RM 247, Lane 5-7: RM270, Lane 8-10: RM28102, Lane 11-13:RM277, Lane 14-16:RM3326, Lane 17-19:RM519, Lane 20-22:RM463, Lane 23-25: RM511

Each marker represents three lanes amplified from DNA of At354 parent, Bg352 parent and RIL 4, respectively

Table 4.17. Probability values resulted from single marker analysis at RM1261 in chromosome 12.

Trait[a]	P value
RL	0.739
SL	0.520^{ns}
RDW	0.092^{ns}
SDW	0.396
SSI	0.520^{ns}
SNK	0.954^{ns}

[a]Root length(RL), Shoot length (SL), Root dry weight (RDW), Shoot dry weight (SDW), Salinty Survival Index (SSI), Shoot Na^+ concentration (SNC), Shoot K^+ concentration (SKC) and Shoot Na^+/K^+ (SNK) ratio,

^{ns}*Not significant*

4.17. Analysis of extremely tolerant and susceptible RILs for other chromosome.

Polymorphism between parents for other chromosomes were carried out with 40 molecular markers. Six polymorphic SSR markers RM13197, RM20224, RM11, RM228, RM24909, RM229 located in chromosome 2, 6, 7, 10 and 11 were found polymorphic between parents in 3% agarose (Figure 4.34) (Table 4.18). And another 2 markers out of 5 were polymorphic for parents in 6% PAGE (Figure 4.35). Twnety percent polymorphic marker percentage was observed in chromosome 2 and 7 (Table 4.22). thirty three percent plymorphic marker percentage was observed in each chromosome chromosome 6,8 and 10. Chromosome 11 showed 16% polymrphic markers from the total markers analysed. .

4.18. Genotyping of extreme RILs for polymorphic markers in other chromosomes

To reduce the cost of experiment, 10 extreme RILs from each of both tolerant and susceptible ends were selected based on SSI and those RIL lines were used to genotype rest of the polymorphic markers scattered through the other chromosomes of the genome. Six markers which were polymorphic in agarose were used to genotype two extreme RILs. Distribution of At354 (At) and Bg352 (Bg) alleles with 6 polymorphic markers among extreme RILs are shown in Table 4.19. The results of students' t- test revealed that there was no significant association observed for any of the traits (Table 4.20), indicating absence of QTLs at those SSR makers.

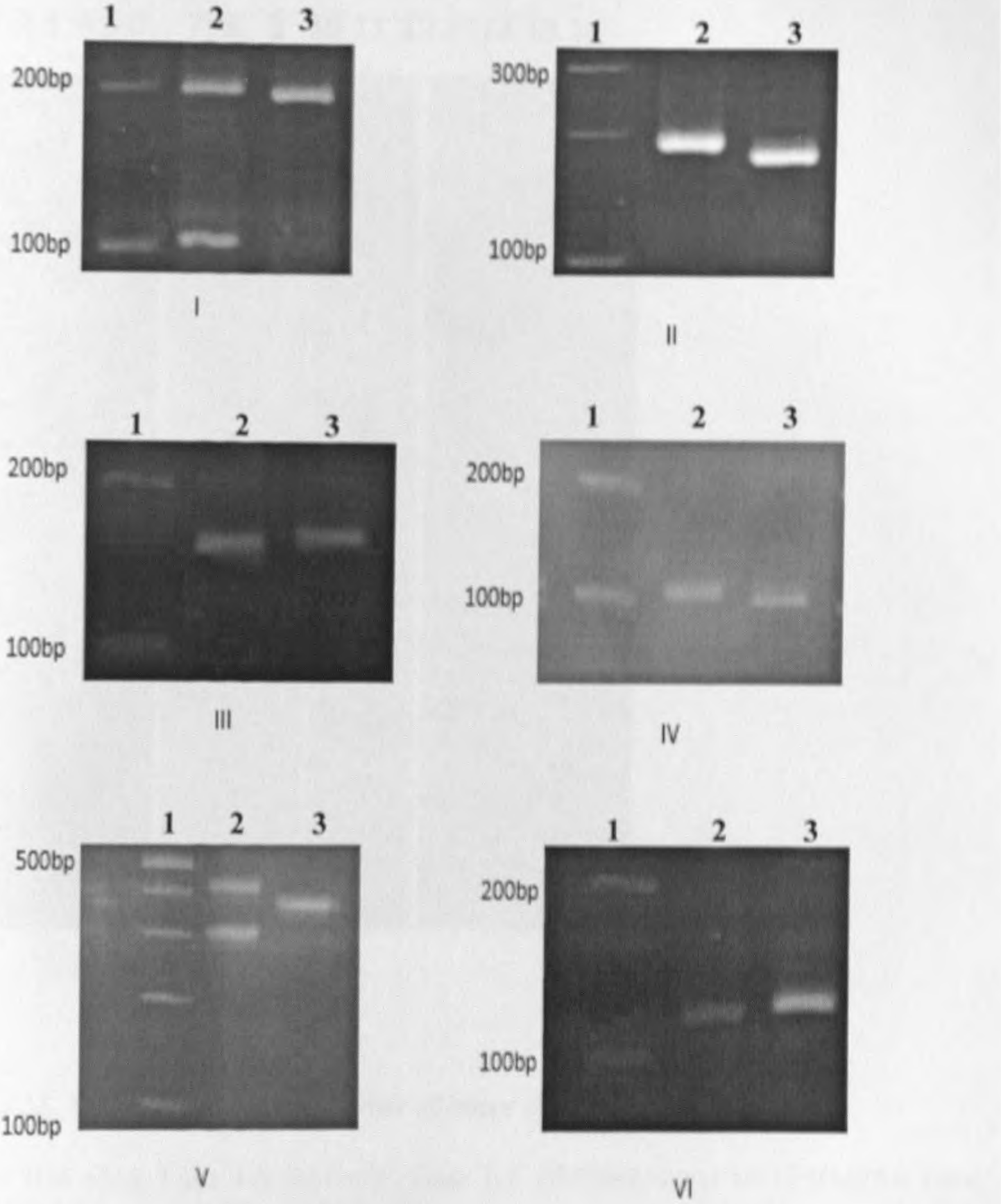


Figure 4.34 PCR amplification with RM13197, RM20224, RM11, RM228, RM24909, RM229 SSR markers located on chromosomes 2, 6, 7, 10 and 11 respectively, showing polymorphism between At354 and Bg352 parental lines

I. RM13197 II . RM20224 III . RM11 IV. RM228 V. RM24909 VI. RM229

Lane 1-100bp DNA ladder, Lane 2-At354, Lane 3-Bg352

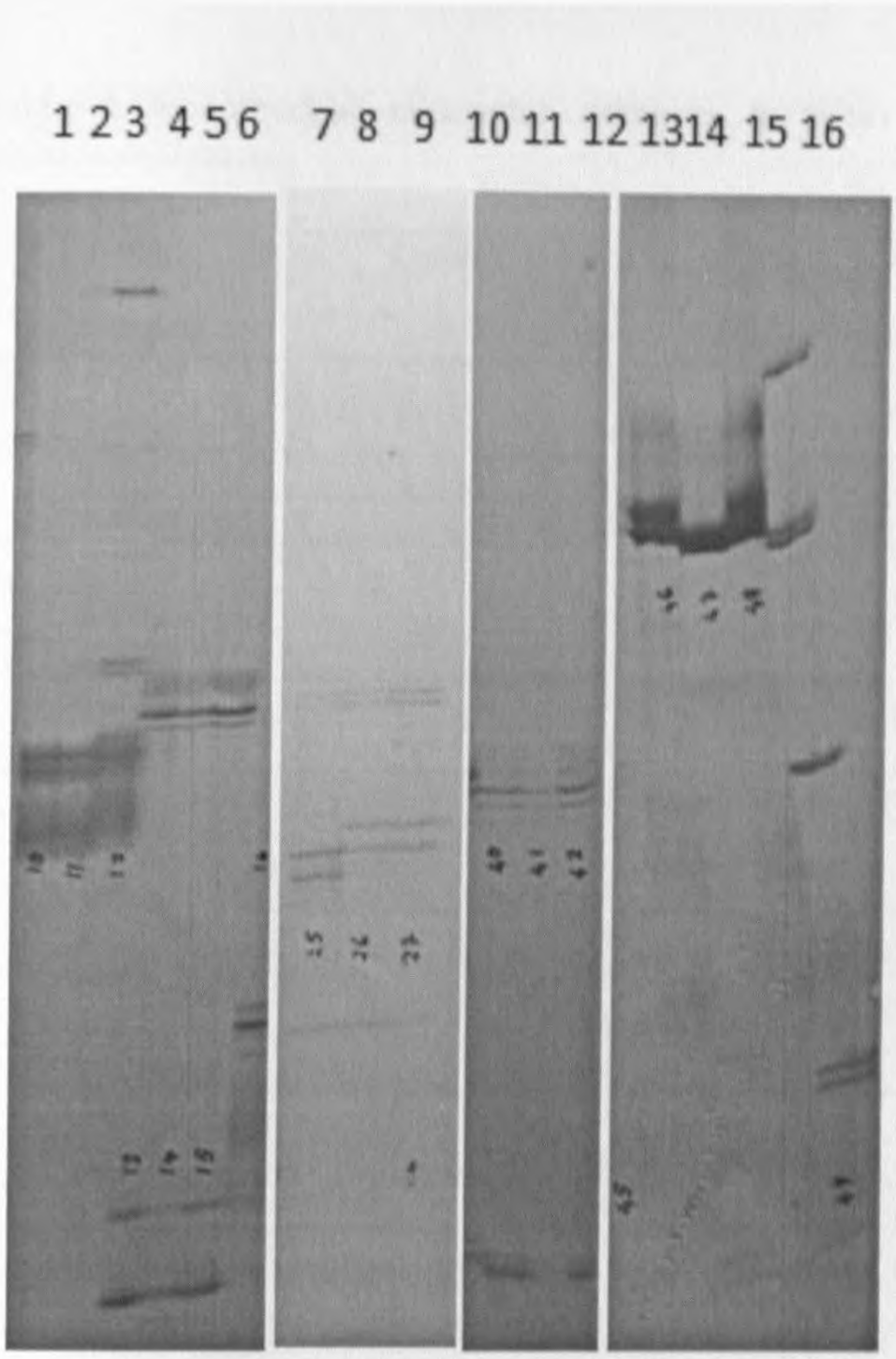


Figure 4.35. Polyacrylamide gel profile of other chromosomes markers

Lane1-3: RM 6318, Lane 4-6: RM5436, Lane 7-9, RM7076, Lane 10-12:RM234, Lane 13-15:RM149, Lane 16:100bp DNA ladder

Each marker represents three lanes amplified from DNA of At354 parent, Bg352 parent and RIL 4, respectively

Table 4.18. Surveyed molecular markers in other chromosomes and their polymorphic status.

	Primer	Chr	Presence of polymophism (P/M) in 3% Agarose	Presence of polymorphism (P/M) in 6% PAGE
1	RM13197	2	**P**	*
2	RM300	2	M	*
3	RM6318	2	M	M
4	RM5404	2	M	*
5	RM154	2	M	*
6	R5M30	5	M	*
7	RM 527	6	M	*
8	R6M14	6	M	*
9	RM20224	6	**P**	*
10	RM248	7	M	*
11	RM7076	7	M	**P**
12	RM11	7	**P**	*
13	RM234	7	M	M
14	RM20848	7	M	*
15	RM21539	7	M	*
16	RM214	7	M	*
17	RM3635	7	M	*
18	RM10	7	M	*
19	RM5436	7	M	M
20	RM5353	8	M	*
21	RM149	8	M	**P**
22	RM25	8	M	*
23	RM242	9	M	*
24	RM7175	9	M	*
25	RM296	9	M	*
26	RM201	9	M	*
27	RM3164	9	M	*
28	RM24330	9	M	*
29	R10M30	10	M	*
30	RM304	10	M	*
31	RM228	10	**P**	*
32	RM258	10	M	*
33	RM24855	10	M	*
34	RM24909	10	**P**	*
35	R11M23	11	M	*
36	RM229	11	**P**	*
37	RM21	11	M	*
38	RM209	11	M	*
39	RM224	11	M	*
40	RM332	11	M	*

Chr- Chromosome
*Not checked in Polyacrylamide gel electrophoresis
P- polymorphic markers between parents, M- Mormorphic markers between parents

Table 4.19. Distribution of At354 (At) and Bg352(Bg) alleles among extreme RILs of 6 polymorphic markers for Salinity Survival Index (SSI).

Marker	RM13197	RM20224	RM11	RM228	RM24909	RM229
Chromosome	2	6	7	10	10	11
RILs with Lowest SSI						
1	At	Bg	At	Bg	Bg	Bg
2	At	At	At	Bg	Bg	At
3	At	At	At	Bg	At	At
7	At	At	At	Bg	At	At
8	At	Bg	At	Bg	Bg	Bg
9	At	At	At	Bg	Bg	Bg
12	At	At	At	At	Bg	At
20	At	At	At	Bg	Bg	Bg
21	At	Bg	At	Bg	Bg	Bg
66	At	At	At	Bg	At	At
RILs with highest SSI						
23	At	Bg	At	Bg	Bg	At
34	At	Bg	At	Bg	Bg	Bg
36	At	At	At	Bg	Bg	At
41	At	At	At	At	Bg	At
72	At	At	At	Bg	Bg	Bg
90	At	At	At	Bg	Bg	At
97	At	Bg	At/Bg	Bg	Bg	Bg
110	Bg	At	At	Bg	Bg	At
111	At	At	At	Bg	At	Bg
163	At	At	At	Bg	At	At

At- At354 allele, Bg- Bg352 allele

Table 4.20. Probability values resulted from single marker analysis of polymorphic markers in other chromosomes

Trait[a]	RM20224	RM228	RM24909	RM229
RL	0.263^{ns}	0.787^{ns}	0.898^{ns}	0.723^{ns}
SL	0.623^{ns}	0.520^{ns}	0.418^{ns}	0.192^{ns}
RDW	0.110^{ns}	0.673^{ns}	0.571^{ns}	0.237^{ns}
SDW	0.819^{ns}	0.517^{ns}	0.960^{ns}	0.739^{ns}
SSI	0.313^{ns}	0.950^{ns}	0.824^{ns}	0.730^{ns}
SNK	0.129^{ns}	0.930^{ns}	0.051^{ns}	0.143^{ns}

[a]Root length(RL), Shoot length (SL), Root dry weight (RDW), Shoot dry weight (SDW), Salinty Survival Index (SSI), Shoot Na^+ concentration (SNC), Shoot K^+ concentration (SKC) and Shoot Na^+/K^+ (SNK) ratio,

[ns]*Not significant*

Table 4.21. Probability values resulted from single marker analysis of RM24909 in chromosome 10.

Trait[a]	P value	
RL	0.205	*ns*
SL	0.696	*ns*
RDW	0.401	*ns*
SDW	0.947	*ns*
SSI	0.591	*ns*
SNK	0.339	*ns*

[a]Root length(RL), Shoot length (SL), Root dry weight (RDW), Shoot dry weight (SDW), Salinity Survival Index (SSI), Shoot Na^+ concentration (SNC), Shoot K^+ concentration (SKC) and Shoot Na^+/K^+ (SNK) ratio,

[ns]Not significant

Table 4.22. polymorphic percentages observed in 12 chromosomes.

Chromosome	No. of surveyed markers	No. of polymorphic markers	Polymorphic percentage %
1	57	18	31.5
2	5	1	20
3	22	7	31.8
4	26	10	38.4
5	1	-	0
6	3	1	33
7	10	2	20
8	3	1	33
9	6	-	0
10	6	2	33
11	6	1	16
12	13	2	26

4.19. Identification of RILs acquiring favorable alleles

As this study was conducted in elite rice background it is worth to select best performing lines and analyze them further. Therefore, RILs that have accumulated promising QTLs above 2 LOD are indicated in Table 4.23. In these QTLs, At354 allele was considered as the favourable allele at peak and the flanking markers considering the additive effect of each QTL.

Table 4.23. RILs containing favorable alleles for promising QTLs

QTL	Chr	Favorable allelic composition at each QTL locus			RIL No.												
		FM1	PM	FM2	72	77	80	84	85	90	107	109	111	114	117	145	149
qSSI1	1	(RM140) A	(RM10793) A	-													
qSL1	1	(RM140) A	(RM10793) A	-													
qSSI4	4	(RM3843) A	-	(RM280) A													
qSL4	4	(RM3843) A	-	(RM280) A													
qSNK4	4	(RM518) A	-	-													
Total number of accumulated QTL Loci					**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Salinity Survival Index (SSI)					0.869	0.653	0.791	0.623	0.655	0.769	0.580	0.683	0.937	0.901	0.508	0.739	0.760

Chr- Chromosome
FM1- Left flanking marker FM2- Right flanking marker PM- Peak marker
A- At354 allele, B- Bg352 allele

5. DISCUSSION

5.1. Hybridization and Development of mapping population

In the present study, At354 improved rice variety was selected as a donor of the salinity tolerant QTLs as it is a one of the cultivars which possesses extreme tolerance to salinity under elite rice background in Sri Lanka. As At354 was derived from the Pokkali which is a salt tolerant landrace extensively used by breeders it was assumed that At354 contain many salinity tolerant QTLs.

Usually QTL mapping needs divergent parents and therefore, Bg352 was selected as the salinity susceptible parent based on the previous studies conducted by Sirisena et al., (2010) and Dahanayake et al., (2015). However, selecting both improved varieties may not be favorable for the role of mapping which needs high percentage of polymorphism between parents. The more the parental lines differ, the more genetic factors can be described or mapped theoretically. However, in this study we selected both elite parents aiming at not only mapping but also directly using the homozygous salinity tolerant recombinants for releasing as salt tolerant lines. Therefore, selection of parental varieties was not purely based on divergence of the varieties as reported in other QTL mapping studies.

Use of molecular marker for testing genetic purity substantially reduce the time, space, labour and ultimately the cost involved in testing the genetic purity of hybrid seeds (Nandakumar et al., 2004; Sundaram et al., 2007). Therefore, genetic purity analysis through SSR marker will be a useful tool for resolving the problem arises in seed certification programme as well as the rapid determination of genetic purity of the rice hybrids. According to the molecular assessment employed in this study, it was able to precisely identify the true hybrids out of 14 F_1 individuals using RM1287 and RM493 (located on chromosome 1 closer to the *Saltol* QTL) polymorphic SSR markers between parents. It was found that 8 lines were not hybrids from the DNA analysis. Hence, it is worth to utilize RM1287 and RM493 markers for verification of hybridity in future breeding programmes which utilize same parental lines.

This study produced a mapping population, by selfing of selected 6 true hybrids and by advancing of subsequent generations upto F_5 generation by single seed descent method representing the recombinant inbred line (RILs) population. Recombinant inbred lines (RILs) are one of the most extensively used mapping populations in rice. Since RILs are developed by single seed descent method, they maintain the early genetic variability as that in F_2.They are the recombinant output from which superior stabilized segregants can be directly used as breeding lines. They serve as means for obtaining recombinants with desirable traits from both the parents involved in the cross and also transgressive segregants of superior quality. From the molecular perspective, RILs serve as a good mapping population. Therefore, mapping and dissecting favourable genes could be carried out at any time, as the population revealing different recombination events and being homozygous for all the allelic loci.

Usually homosygosity in F5 population is considered as more than 90%. Therefore, in this project QTL mapping was conducted with F5 without waiting for F6. However, some lines were advanced to F6 as it was a project requirement. According to Vinod, 2006, RIL population used for mapping in this study ($F_{5:6}$) should possess around 92.25 within line percentage homozygosity at each locus as described in Table 5.1 quoted

from Vinod, 2006. However, in the proposal of this study, it was planned to continue QTL mapping using F2.3 families. Due to the fact that the study commenced late, it was able to produce F5 progeny and therefore, this study was proceeded with F5 lines. The results of the ANOVA also proved that within line variation in F5 was negligible.

Table 5.1. Level of inbreeding and corresponding percentage within-line homozygosity at each locus in RIL population

RIL population level of inbreeding	Percentage within-line homozygosity at each locus
$F_{3.4}$	75.0
$F_{4.5}$	87.5
$F_{5.6}$	92.25
$F_{6.7}$	96.875
$F_{7.8}$	98.4375
$F_{8.9}$	99.21875

Source: Vinod, 2006

5.2. Variation of phenotypic traits in mapping population under salinity stress

Results of this study revealed significant differences between two parental genotypes, At354 and Bg352 for all the traits assessed in this study exhibiting divergent performances of two parents under salinity stress condition proving their suitability to construct mapping population for salinity tolerance.

Eventhough, studied mapping population exhibited varying degree of visual salt injury symptoms, standard evaluation score (SES) was assigned as a categorical data based on the extent of visual symptoms forming discrete classes as 1,3,5, and 9. Therefore, SES was not assessed by parametrical analysis thereby could not be included in the QTL analysis. However, using SES it is possible to assess the population initially with the discrimination of susceptible and tolerant lines providing the basic idea of the nature of the population before leading to further assessments.

Thus, in present study it was realized the importance of assessing survival potential of RILs under salt stress with a quantitative parameter as such parameter is convenient for the analysis, mapping and independent from personal skills. Accordingly, a quantitative parameter called salinity survival index (SSI) which ranges from 0-1 was designed by giving maximum weight for the plants that survived throughout whole period (SSI =1) while giving minimum weight for the plants that died at earliest possible (SSI closer to 0) (Wijerathna et al., 2014). The SSI is a novel index and results of SSI of this study proved its applicability in all sort of analysis such as frequency distribution, correlation and gene mapping.

Frequency distributions of all measured traits revealed the wider continuous variation of the traits (except SES which was in categorical basis) and they showed comparatively

higher (coefficient of variation) CV. This indicates the occurrence of substantial number of recombinants of parental genotypes with different genetic status within the population which would facilitate the QTL mapping for salt tolerance. Association of higher CV with dry biomass of shoot (CV-34.99%) and root (CV- 47.48%) showed comparatively wider variation than the variation of shoot and root length among RILs under salt stress. Thus, it could be suggested that comparatively higher number of genes might be involved in the differential expression of shoot and root biomass under salt stress compared to shoot and root elongation.

The existence of individuals that acquiring exceeded phenotypes than their parents appear in a segregating population is known as transgressives (Mauricio, 2001). In our study also we examined many transgressive RILs for both the direction and this might be due to the inheritance of alleles from both the parents.

Salinity tolerance is a collection of physio-morphological mechanisms. Using methods like SSI, plant height, bio mass and shoot Na+/K+ the mechanisms which generate the tolerance of rice seedlings towards salt can be measured. According to the table 4.4 the traits resulted from our study showed many correlations among themselves, probably may be due to their inter-related salinity tolerance mechanism. These observations were more strengthened with the results of the analysis of variance (ANOVA) which revealed the highly significant differences among RILs ($P<0.001$) for all the measured traits.

5.3. Mapping of QTLs for salt tolerance

In this study, SSR markers were mainly used for the genotyping of mapping population due to their abundance, high efficiency, reproducibility, easy-to-use, co-dominance nature and generation of high degree of polymorphism in rice. However, in this study according to the parental polymorphism survey comparatively low percentage of polymorphism was detected in SSR loci. Since both parents have background of Bg varieties in their pedigree, low rate of polymorphism may be due to sharing of elite characters of Bg varieties in both parents eventhough they exhibited divergent performances under salinity stress condition. Total polymorphism observed in At354 and Bg352 cross was less compared with some of the reported studies such as Lang et al. (2008) and Islam et al., (2011). However, study conducted by Luu et al., (2012) reported that the total polymorphic markers appeared in AS996/FL478 cross was only 12.6%.

Previously, several mapping studies have identified QTLs associated with salinity tolerance in rice (Ammar et al., 2007; Bonilla et al., 2002; Flowers et al., 2000; Gregorio, 1997a ; Haq et al., 2010; Kim et al., 2009; Koyama et al., 2001; Lee et al., 2007; Lin et al., 2004; Prasad et al., 1999; Sabouri et al., 2009; Singh et al., 2007; Singh and Flowers 2010; Takehisa et al. ,2004). A study employing an $F_{2:3}$ population between the tolerant *indica* landrace Nona Bokra with the susceptible *japonica* Koshihikari identified several QTLs controlling tolerance traits, including major QTLs for shoot K^+ concentration on chromosome 1 (*qSKC-1*) and shoot Na^+ concentration on chromosome 7 (*qSNC-7*) (Lin *et al.*, 2004). Similarly, a recombinant inbred line (RIL) population between the highly tolerant landrace Pokkali and sensitive IR29 identified a major QTL designated as *Saltol*, on chromosome 1 at the 10.8 -16.4 Mb, in the same region as *qSKC1*, explaining 43% of the variation for seedling shoot Na^+/K^+ ratio (Gregorio, 1997a; Bonilla *et al.* 2002). Therefore, it was identified that *Saltol* QTL has significant

impact on seedling stage salinity tolerance. As the RIL population of this study was also derived from parents with the pedigree of Pokkali it was expected to have similar QTL within this region. Therefore, QTL analysis in chromosome 1 was narrowed down to closer region to *Saltol* QTL.

In chromosome 1, 2 linkage groups (7 markers in linkage group 1 and 4 markers in linkage group 2) were resulted covering narrow length of chromosome 1. Even though, preference was to have one linkage map for all 12 markers on chromosome 1, it was not be able to merge these two groups as polymorphic SSR markers were not well distributed across chromosome 1 which could not sufficiently cover the whole length of chromosome. Eleven markers out of 12 were concentrated to the 10.8-14.2 Mb region. which is the region composed of *Saltol* QTL. Semagn, et al., (2006) also reported that possibility of resulting linkage groups higher than the haploid chromosomes if the molecular markers are not well-distributed across all chromosomes and do not sufficiently cover the genome.

In this study, QTL mapping was performed using single marker analysis (student's t-test and single marker regression) and composite Interval mapping. Single-marker analysis is the simplest method to investigate individual markers independently and without reference to their position or order. It assesses the segregation of a phenotype with respect to a marker genotype and indicates which markers are associated with the quantitative trait of interest and, therefore, point out the existence of potential QTL.

In chromosome 1, 11 of such marker- trait associations were detected by simple student's t-test of which 3 traits were associated with 2 marker loci, RM140 and RM10745, 2 traits were associated with RM1287 and single traits were associated with RM10772, RM493 and RM10771 marker loci.

However, single marker regression revealed only 4 marker- trait associations where each of RM140 and RM10745 were associated with 2 traits. Doerge, (2002) reported that single marker approach has the potential to detect numerous significant markers which could include false positives as well, especially with the small size of population. Further, he emphasized that although the t- test, ANOVA and simple linear regression approach are all equivalent to each other when their hypotheses are testing for differences in the phenotypic means, they fail to provide a precise estimation of QTL location, or recombination frequency between the marker and the QTL. This is because the QTL effect and the location are confounded, or are unable to be estimated separately.

Therefore, to overcome such drawbacks, simple interval mapping (SIM) could be employed in QTL analysis which makes use of linkage map and analyses intervals between adjacent pairs of linked markers along chromosomes simultaneously, instead of analyzing single markers (Lander and Botstein, 1989). The use of linked markers for analysis, compensates for recombination between the markers and the QTL, and is considered statistically more powerful compared to single-point analysis (Lander and Botstein, 1989). Other than the SIM, composite interval mapping (CIM) approach could be employed to enhance the power of detection of QTLs more precisely over the other 2 approaches. In this method interval mapping combines with linear regression to include additional genetic markers as cofactors (outside the window of analysis) in the statistical model in addition to an adjacent pair of linked markers for interval mapping, with the

purpose of removing the variation that is associated with other (linked) QTL in the genome (Jansen, 1993; Jansen and Stam, 1994; Zeng, 1993, 1994; Doerge, 2002).

In this study, experiment-wise significance level of LOD threshold was estimated using permutation test (with 1000 repeats) for declaring the significant putative QTL at 0.05 and 0.01 significance level. Before permutation tests were widely accepted as an appropriate method to determine significance thresholds, a LOD score of between 2.0 to 3.0 (most commonly 3.0) was usually chosen as the significance threshold (Collard et al., 2005). It is very important to demarcate a specific statistical threshold LOD value for QTLs to be significant. If the peak of the QTL exceeds the threshold LOD value, it can be declared as a putative QTL. Generally threshold LOD values lie between 2 to 3 (Masood et at, 2004). Many studies showed that threshold LOD value varies according to the experiment conducted (Ahmadi and Fotokian 2011; Ismail et al., 2011 and Thomson et al., 2010).

In this study, according to CIM approach, in chromosome 1, number of significant QTLs was reduced to 3 QTLs and they were named as *qSSI1*, *qSL1* and *qSNK1* according to the nomenclature method described by McCouch and CGSNL (2008). QTLs, *qSSI1* and *qSL1* were co-located at RM10793 at 12.5 Mb and *qSNK1* was located at RM10772 at 12 Mb position accounting substantial proportion of each respective phenotypic variation ranging from 8.9%-10.8%. All 3 potential QTL positions were identified within the region of *Saltol* QTL indicating the probable inheritance of *Saltol* region from the Pokkali background.

In chromosome 3, all five polymorphic markers were linked in a one group. Although markers RM3867, RM7076 and RM3864 were polymorphic in the cross IR29/Pokkali conducted by Thomson et al., (2010), they were monomorphic in our cross At354/Bg352. All SIM and CIM analysis indicated that there is no association between polymorphic markers and the phenotypic traits in chromosome 3. Usually, it is necessary to develop dense linkage maps and to have high number of individuals in the population to detect minor QTLs by mapping. Probably due to less number of polymorphic markers and less number of individuals in the population may be the possible cause for not detecting any significant QTL in chromosome 3.

In chromosome 4, both SIM and CIM verified the presence of QTLs and they were named as *qSL4*, *qSNK4* and *qSSI4*, according to the method described by McCouch and CGSNL (2008). QTLs for shoot length (*qSL4*) and SSI (*qSSI4*) were located in the region of RM3843-RM280 while QTL for shoot Na^+/K^+ (*qSNK4*) was located at RM518 locus. Usually if a QTL explains more than 10% of the phenotypic variation, it can be considered as a major QTL as reported by Collard et al., (2005). Therefore the three QTLs identified in chromosome 4 can be demarked as major QTLs.

QTL for shoot length and SSI had positive additive effect from At354 allele because the At354 allele contributes to increase the measurements of shoot length and SSI by 3.3 and 0.14 units respectively. For Na+/K+ additive effect was negative for At354 allele as the Bg352 allele was the responsible allele for the increment of Na+/K+ in RILs. At354 allele in RIL population contributes to decrease the Na+/K+ by 2.25 units. Thomson et al, (2010) reported a QTL in chromosome 4 for plant height closer to the region reported in this study. The peak of the previously reported QTL by Thomson et al, (2010) lies on RM3843 marker region and in our study peak region was detected at

RM280 (Figure 4.31) which are close to each other. From these evidences, it can be speculated that these two instances refer to the same QTL.

Previous studies showed that the QTL mapping performed with a comparatively small number of individuals in a single environmental condition show only the QTLs which have large effect and unable to detect QTLs those show smaller effects (Tanksley, 1993; Edwards et al., 1992). The number of putative QTLs identified in this study was comparatively low and it might be due to the use of a small population with random selection. However, it has been reported that minimum number of individuals in a segregating population should not be less than 50 for QTL mapping (Collard et al., 2005; Mohan et al., 1997), but for fine mapping a larger population is required. In this study we were able to proceed the project with 100 individuals following the recommendation by Collard et al. (2005) and Mohan et al. (1997). In this study we selected 100 RILs randomly. However, if we had obtained 100 RILs representing extreme ends of the population we would have obtained many QTLs as reported by Collard et al. (2005).

Usually, after mapping QTLs, such QTLs need to be validated and confirmed by repeating the mapping study in different environments and seasons. Then promising QTLs can be used to detect candidate genes or to backcross breeding. Usually, QTL mapping studies are performed with divergent parents where trait-donor -parent derived from not elite genetic background. Therefore, scientists use to backcross the QTL-donor parent to a recurrent parent which possess other favorable agronomic traits to get fewest donor alleles and maximum recurrent alleles by using marker assisted selection. In this study, the both parents, At354 and Bg352 are high yielding varieties and possess elite genetic background. Therefore, RILs that show high salinity tolerance by accumulating investigated QTLs, can be released directly as cultivars if they contain other favorable agronomic traits. At the moment, some RILs that have accumulated promising QTLs and performed best in withstanding salinity are indicated in Table 4.23 and they have been chosen for further analysis for reproductive stage salinity and agronomic traits collaborating with RRDI, Batalagoda as an application of this project.

This study needs further analysis of the genome with some other molecular makers due to the fact that low level of polymorphism was present in SSR markers of this cross. In this regards, this study was extended as an application of the project with SNP markers by collaborating with International Rice Research Institute (IRRI), Philippines. Collaboration with IRRI provided Illumina Infinium rice 6K SNP chip that contained 5,274 SNP loci covering all 12 rice chromosomes. The results revealed that 1137 SNPs markers were polymorphic between At354 and Bg352 and thereby highly dense linkage maps along with QTL maps were produced.Therefore, these highly dense linkage maps can be utilized for mapping of salinity or any trait that show divergence in the parents.

6. CONCLUSIONS AND RECOMMENDATIONS

The project was started by making a successful cross between At354 and Bg352 elite rice varieties of which At354 was used as a donor of the salinity tolerance and female partner of the cross. Hybridity of 6 F1 individuals derived from At354/Bg352 cross was confirmed precisely with the RM1287 and RM493 SSR markers. It was also proved the feasibility of utilizing RM1287 and RM493 markers for verification of hybridity in

future breeding programmes which utilize same parental lines. By selfing of 6 true hybrids, 350 F2 segregants were raised successfully followed by advancing of subsequent generations upto F5 generation which is used for mapping. Finally, it was able to produce a RIL population consists of 281 lines with the expected level of homozygosity, to be used as the mapping population for salinity tolerance.

Nine morpho-physiological traits were assessed in 100 RILs that were randomly selected from 281 RIL population under EC of 12 dSm^{-1} of salinity. Frequency distributions of these 9 morpho-physiological traits *viz.*, standard evaluation score, salinity survival index (SSI), shoot length, root length, shoot dry weight, root dry weight, shoot Na^+ concentration, shoot K^+ concentration and shoot Na^+/K^+ ratio indicated the broad spectrum of genetic variability in the At354 x Bg352 RIL population under salinity stress.

Out of 158 molecular markers, representing entire rice genome, 45 markers showed polymorphism for parental genotypes in 3% agarose and 6% Polyacrylamide gel electrophoresis. The overall polymorphism in the genome of this cross is 28.4% which is relatively low.

According to the composite interval mapping results, three putative QTLs, *qSSI1*, *qSL1* and *qSNK1* were identified responsible for SSI, shoot length and shoot Na^+/K^+ concentration respectively in the chromosome 1. Of them, *qSSI1* and *qSL1* were co-located at RM10793 at 12.5 Mb position and *qSNK1* was at RM10772 at 12 Mb accounting 8.9%-10.8% phenotypic variations. All 3 potential QTL positions were identified within the region of *Saltol* QTL indicating the probable inheritance of *Saltol* region from the Pokkali background. However, in this experiment more precise location of QTLs could not be traced due to the low rate of polymorphism which generated rather low dense map with SSR markers.

Single marker analysis enabled to detect three SSR markers in chromosome 4 that were significantly linked with SSI at RM280, shoot length at RM280 and Na+/K+ at RM518 locations. QTLs identified for both SSI and shoot length were flanked between RM3843 – RM280 markers. Composite interval mapping also revealed the same QTLs on shoots length ($P<0.05$), Na+/K+ ($p<0.001$) and SSI ($P< 0.01$) and they were named as *qSL4*, *qSNK4* and *qSSI4* respectively. Identified QTLs, *qSL4, qSNK4* and *qSSI4* were able to explain 11%, 16% and 15% of phenotypic variation respectively. Linkage map was constructed for chromosome 3 from 5 polymorphic markers. Results obtained from single marker analysis for each marker location and composite interval mapping did not show any association between marker genotype and phenotypic traits in chromosome 3. Another 6 polymorphic markers were found in chromosome 2,6,10 and 11 and genotypic data obtained from those markers did not reveal significant association with phenotypic traits under salt stress.

The RILs which contained 5 promising QTLs with above 2 LOD *viz.*, *qSSI1, qSL1, qSL4, qSNK4* and *qSSI4* were identified from the RIL population and they were selected for further analysis on salinity tolerance in reproductive stage and favourable agronomic traits as an application of this project.

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8. PROBLEMS IF ANY, ENCOUNTERED DURING THE COURSE OF THE PROJECT

The major problem encountered in this study was the presence low polymorphism in SSR loci of the two parents. However, most of the previous studies of rice conducted with SSR markers have not mapped their genes/QTLs with more than 200 SSR. Theretofore, it is unlikely to get a saturated linkage map with SSR markers which produce polymorphism maximum with few hundreds. As a solution to this scientists have developed high throughput SNP technology which usually produce polymorphism with more than 1000 markers. Therefore, SNP based linkage mapping definitely produce highly saturated maps in rice mostly producing less than 1cM between two adjacent markers, thereby useful to be proceeded to candidate gene discovery even.

Therefore, in this project as a solution to the problem of low polymorphism, we extended this study with SNP analysis collaboratively with IRRI, Philippine. The results of SNP based QTL mapping revealed many promising QTLs presenting between the flanking markers of less than 1Mb intervals in At354x Bg352.

9. MAJOR FINDINGS

Six QTLs for salinity tolerance, namely, *qSSI1*, *qSL1, qSNK1, qSSI4, qSL4 and qSNK4,* inherited from At354 allele were found in At354 x Bg352 mapping population.

Section 4

Impact of Research results:

(a) i. Relevance of results achieved to scientific advancement
ii Dissemination/application of research output – please give the tentative plan

(b) i Relevance of results achieved to national/socio-economic development - please give recommendations clearly as this write – up may be forwarded to the potential users who will benefit from the research

Relevance of results achieved to scientific advancement:

a) i.

1. In this study, it was able to produce a RIL population consisted of 281 lines with the expected level of homozygosity, to be used as the mapping population for salinity tolerance.

2. According to the composite interval mapping results, three putative QTLs *viz.*, *qSSI1*, *qSL1* and *qSNK1* were identified responsible for SSI, shoot length and shoot Na^+/K^+ concentration respectively in the chromosome 1 while another three putative QTLs *viz.*, *qSSI4, qSL4* and *qSNK4* responsible for SSI, shoot length and shoot Na^+/K^+ concentration traits respectively were identified in chromosome 4. The locations of these QTLs would be useful to introgress into other varieties by backcross breeding to make them salt tolerant.

3. To the best of our knowledge, in Sri Lanka, At354 x Bg352 has been the first mapping population reported and our QTL maps have been the first QTL maps developed from a native mapping population. This project revealed the complete procedure of QTL mapping starting from the initial stage of hybridization thereby useful to be applied for any self pollinated annual crop to map the genes/QTLs following similar procedure.

4. Also this project has put the foundation step to utilize high throughput SNP technology which is a kind of scientific advancement. This was achieved by a lecturer (Prob) attached to Wayamba University of Sri Lanka (WUSL) who had registered for a PhD in WUSL, as she got a scholarship to work with At354 x Bg352 mapping population in the International Rice Research Institute, Philippines. With this project she was able to develop highly dense linkage maps by employing high-throughput SNP marker technology (Illumina Infinium rice 6K SNP chip designed by Mark Wright and Susan McCouch, Cornell University). Accordingly, highly saturated genetic map of 12 chromosomes spanning 1460.81 cM of the rice genome was generated by 1135 polymorphic SNP markers. The results revealed promising 17 QTL hotspots governing 2-10 multiple traits related to salinity stress which has been a scientific advancement.

ii) Dissemination/application of research output – please give the tentative plan:

1. We have published papers and abstracts and all of them have been listed in Section 1, Xiii (from Annexure 4 to Annexure 14)

2. A workshop on "QTL/ gene mapping in plants" was conducted on 8th-9th September 2015 at Wayamba University of Sri Lanka to disseminate the knowledge that was obtained from this project. There were 22 participants representing various institutes of Sri Lanka. Brochure of the workshop (Annexure 15), Program of the workshop (Annexure 16), list of participants (Annexure 17) and participants' attendance list (Annexure 18) are attached.

3. This research project was able to be extended to analyze the genome of At354 x Bg352 with SNP markers via Global Rice Science Scholarship program. Therefore, as an application of the proposed study, it was able to develop highly dense linkage maps by employing high-throughput SNP marker technology (Illumina Infinium rice 6K SNP chip designed by Mark Wright and Susan McCouch, Cornell University). Accordingly, highly saturated genetic map of 12 chromosomes spanning 1460.81 cM of the rice genome was generated by 1135 polymorphic SNP markers. The results revealed promising 17 QTL hotspots governing 2-10 multiple traits related to salinity stress. Also these SNP loci would be important in future studies in candidate gene discovery for salt tolerance because most of the promising QTLs investigated in this extended study could be detected in between the flanking markers of less than 1 Mb intervals in At354x Bg352. Presently, a manuscript is being written with all these information.

4. From this project (including the extended part conducted at IRRI) we have recognized few promising RIL lines that have accumulated all salinity tolerant QTLs along with better phenotypic performance under salinity stress. We have planned to handover the above RILs to Rice Research and Development Institute, Batalagoda to analyze those selected RIL lines for agronomical traits, reproductive stage salinity tolerance and coastal salinity tolerance etc. In this regard a discussion was held on 19th August 2015 with the Director/ Rice Research and Development Institute, Batalagoda and the Director agreed to proceed the work.

b) i. Relevance of results achieved to national/socio-economic development - please give recommendations clearly as this write – up may be forwarded to the potential users who will benefit from the research

1. This project made the avenue to a Sri Lankan student (Ms DR Gimhani who is a lecturer attached to WUSL) to extend the same study (work with the cross of At354 x Bg352) to a PhD at IRRI by receiving Global Rice Science scholarship (Annexure 19)

2. This research project developed the human capacity in the area of plant molecular breeding by producing one M.Phil degree and one PhD degree. Details are as follows.

   M.Phil Degree: Ms Buddhika Dahanayake who worked as the research assistant for the project completed an MPhil degree under the title of "Molecular mapping of

quantitative trait loci associated with salinity tolerance using a RIL population of rice" in WUSL. She already submitted the thesis and defended the thesis on 25th August 2015 (Annexure 1 and 2)

PhD Degree: Ms DR Gimhani, registered for a PhD in WUSL and completed two chapters of her study, 1. Development of mapping population with At354 x Bg352 and 2. Mapping for salinity tolerant QTLs in chromosome 1 under the support of NSF/BT/2011/02 grant. She completed rest of the chapters of PhD with the support of Global Rice Science Scholarship in International Rice Research Institute, Philippines. She already submitted the completed thesis to WUSL on 25th August 2015 and is waiting for defense examination (Annexure 3).

3. This research project has launched foundation steps to develop salt tolerant varieties with the help of Rice Research and Development Institute (RRDI), Batalagoda.

   As the overall impact of the QTL study, it could be suggested that chromosomal regions/loci possessing different QTLs provide an opportunity for breeders to introgress such regions together as a unit into elite rice varieties through marker assisted selection (MAS)/ marker assisted backcrossing (MAB) and to develop resilient salt-tolerant cultivars. Also, this research produced few RILs that showed best performance on seedling stage salinity tolerance and accumulation of investigated QTLs. These lines are planned to be submitted to Rice Research and Development Institute, Batalagoda to analyze them for agronomical traits, reproductive stage salinity tolerance and coastal salinity tolerance etc. In this regard a discussion was held on 19th August 2015 with the Director/ Rice Research and Development Institute (RRDI), Batalagoda and the Director/ RRDI agreed to proceed with necessary trials.

4. This research project created enormous knowledge in QTL mapping and this knowledge was disseminated to Sri Lankan scientists via a 2 day National workshop.

   Although Sri Lanka has many divergent crop germplasm, these germplasms have not been utilized much in modern breeding programs mainly because their genetic status is unrevealed to the world. QTL mapping based approach is one of promising ways of finding out genes for many traits such as yield, quality and abiotic and biotic stress tolerance etc. Therefore we organized a workshop with the long term objective of utilizing favorable genes of divergent germplasm in Sri Lanka for breeding of improved varieties. In this regard we invited students, researchers, breeders or any beginner of this subject to gain the knowledge on QTL/Gene mapping in simplified level so that they can make use of this knowledge directly in breeding purposes for crop improvements, proposal preparation to obtain research grants and for initiation of collaborative research programs etc. The information of the workshop (Brochure of the workshop (Annexure 15), Program of the workshop (Annexure 16), list of participants (Annexure 17) and participants' attendance list (Annexure 18)) are attached for the information.

Section 5

Miscellaneous

i. List of major equipment acquired during the project period, their value and their functionality -Nill

ii. List of publications/communications arising from the project and/or presentations made at seminars, workshops etc. (Please attach copies)
List of publication was mentioned in Section 1 –Xiii (Annexure 4 to 14). Workshop information is attached in Annexure 15 to 18.

Section 6

Summary Statement of Expenditure (indicate under Personnel, Equipment, Consumables, Lab services & sample analysis, Statistical analysis, Calibration of instruments, PG registration fee, Travel and Subsistence and Miscellaneous)

Financial report is attached in Annexure 20.

Section 7

i. Signatures of Investigators (PI as well as Co-Is)

.....

Prof.N.S.Kottearachchi
Professor
Department of Biotechnology
Faculty of Agriculture &
Plantation Management
Wayamba University of Sri Lanka

Principle Investigator:
Dr. N. S. Kottearachchi, Professor, Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka.

.....

Dr. W.L.G. SAMARASINGHE
Assistant Director of
Agriculture (Research)
Plant Genetic Resources Centre
Gannoruwa, Peradeniya.

Co Investigator:
Dr. W. L. G. Samarasinghe, Assistant Director of Agriculture
Former address: Biotechnology Unit, Rice Research and Development Institute, Department of Agriculture, Batalegoda, Ibbagamuwa.

ii. Comments of the Head of the Department/signature

Satisfactory.
21/09/15

Dr. (Mrs.) Kalaivani Vivehananthan
Head
Department of Biotechnology
Faculty of Agriculture & Plantation Management
Wayamba University of Sri Lanka
Makandura, Gonawila (NWP)
Mob : + 94 773988612

iii. Head of the Institution's signature

VICE-CHANCELLOR
WAYAMBA UNIVERSITY OF SRI LANKA
KULIYAPITIYA

Annexure 01

ශ්‍රී ලංකා වයඹ විශ්වවිද්‍යාලය
කුලියාපිටිය

WAYAMBA UNIVERSITY OF SRI LANKA
KULIYAPITIYA

දුරකථන අංක Telephone Nos : උපකුලපති Vice Chancellor :037-2282758 ලේඛකාධිකාරී Registrar :037-2283165 මූල්‍යාධිකාරී Bursar :037-2284168 අධ්‍යයන අංශය Academic Branch :037 2284374 පොදුව General :037 2281412
ෆැක්ස් /Fax 037-2281392 :037-2283165

මගේ අංකය : My Number :
ඔබේ අංකය : Your No :
දිනය : Date : 24.04.2015

TO WHOM IT MAY CONCERN

Status of Master of Philosophy – Ms. BA Dahanayaka

This is to inform that Ms. BA Dahanayaka has been registered for a Master of Philosophy Degree at the Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka with effect from 27.03.2013 under the registration number WU/SRHDC/MPHIL/2013/25, and has successfully finished and submitted her M. Phil Master thesis to the Academic Branch by 24.04.2015.

Title of the M.Phil study - "Molecular mapping of quantitative trait loci associated with salinity tolerance using a RIL population of rice".

Student Status - Full time.

This letter is issued on the request of Ms. BA Dahanayaka.

Thank You

Registrar

REGISTRAR
WAYAMBA UNIVERSITY OF SRI LANKA
KULIYAPITIYA.

Annexure 02

ශ්‍රී ලංකා වයඹ විශ්වවිද්‍යාලය
කුලියාපිටිය
Wayamba University of Sri Lanka
Kuliyapitiya

දුරකථන අංක : උපකුලපති : 037-2282758 ලේඛනාධිකාරී : 037-2283165 මුලාධිකාරී: 037-2283168 කාර්යාල : 037-2281412
Telephone Nos. : Vice Chancellor : Registrar Bursar General : 037-2281414
: 037-2283614

ෆැක්ස් : : 037-2281392 : 037-2283165 037-2283168
Fax

මගේ අංකය :- SRHDC/MPHIL/2013/25 ඔබේ අංකය : දිනය:. 13.08.2015

My Number Your Number Date ;

Ms. BA Dahanayaka,
17/1, Palpola,
Kahawala,
Padukka

Dear Ms. Dahanayaka,

Thesis Defense Examination

This is to inform you that the Thesis Defense Examination of your research, related to the Master of Philosophy Degree of the Wayamba University of Sri Lanka, is scheduled to be held on 25/08/2015 at 10.00 a.m. at the Board Room of the Faculty of Agriculture & Plantation Management, Wayamba University of Sri Lanka, Makandura.

You are expected to make a presentation of 30 to 45 minutes of your research in front of the Panel of Examiners. Please be present yourself for this examination without any failure.

13/08/2015

Senior Assistant Registrar/ Examinations

P. K. SENEVIRATNE
Senior Assistant Registrar/Examinations
Wayamba University of Sri Lanka
KULIYAPITIYA.

Cc. 1) Chairman /SRHDC
2) Assistant Registrar / Academic Branch

Annexure 03

Wayamba University of Sri Lanka
Kuliyapitiya, Sri Lanka
Office of the Registrar

My Number | Your No | Date of the Letter

Managing Director,
Global rice Science Scholarship 2011,
International Rice Research Institute (IRRI),
Philippines.

Dear Sir/Madam,

Submission of Ph.D Thesis-Ms. DR Gimhani (WU/SRHDC/PHD/2011/08)

This is to confirm that Ms. DR Gimhani (WU/SRHDC/PHD/2011/08) has submitted her Ph.D thesis titled "Identification of salinity tolerant QTLs in elite rice background" to the Wayamba University of Sri Lanka by 25.08.2015.

Thank You,

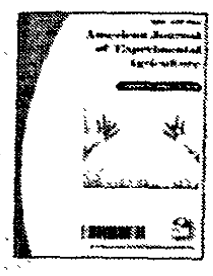
Yours' sincerely,

Registrar

G Piyaratne
Registrar
Wayamba University of Sri Lanka
Kuliyapitiya.

Tele: +94(0)37 22 83165 Fax: +94(0)37 22 84663 e-mail: registrar@wyb.ac.lk

Annexure 04



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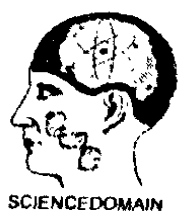

Assessment of Salinity Tolerance and Analysis of SSR Markers Linked with *Saltol* QTL in Sri Lankan Rice (*Oryza sativa*) Genotypes

B. A. Dahanayaka[1], D. R. Gimhani[1], N. S. Kottearachchi[1*] and W. L. G. Samarasighe[2]

[1]Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba Univesity of Sri Lanka, Makandura, Gonawila (60170) (NWP), Sri Lanka.
[2]Rice Research and Development Institute, Batalagoda, Ibbagamuwa, Sri Lanka.

Authors' contributions

This work was carried out in collaboration between all authors. Author BAD conducted the experiment, involved in data collection, analysis, and interpretation and wrote the first draft of the manuscript. Author DRG established the experimental design, involved in data collection, analysis and revision of the first draft of the manuscript. Author NSK guided and reviewed the experimental design and all drafts of the manuscript. Author WLGS guided and facilitated the collecting of required planting materials. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aim: This research was aimed at assessing Sri Lankan rice varieties for the salinity tolerance using morphological traits and to analyze the SSR markers closer to *Saltol* QTL of the chromosome 1 to be used in rice breeding and gene mapping studies.
Place and Duration of Study: The research was conducted at the Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, from April 2013 to October 2013.
Study Design: Morphological traits were analyzed by ANOVA and polymorphic bands obtained from SSR markers were analyzed by Jaccard's similarity coefficient following the unweighted pair group method with arithmetic mean (UPGMA).

**Corresponding author: E-mail: kottearachchins@yahoo.com;*

Methodology: Morphological traits of twenty rice germplasm of Sri Lankan origin, including traditional and improved varieties were assessed under 12 dS/m saline stress and five SSR markers located between 10 -15 Mb to *Saltol* QTL were analyzed in relation to salinity tolerance
Results: A novel weighted indicator, Salinity Survival Index, revealed that Goda Wee, At354 and Al Wee varieties were highly salinity tolerant compared to the tested varieties including an accession of Pokkali and the morphological traits also showed the same validation. Diversity analysis of SSR marker alleles linked with *Saltol* QTL clustered the salinity tolerant and salinity susceptible germplasms as compatible with the distribution pattern of salinity survival index and the marker, RM1287 was more informative for the screening of rice germplasm for salinity tolerance
Conclusion: Information derived on morphological traits under salinity stress and the polymorphic SSR marker patterns obtained from tolerant and susceptible varieties near *Saltol* region would be useful in selecting parental lines from the tested varieties for rice breeding and gene mapping programs designed for salt tolerance.

Keywords: Rice germplasm; salinity tolerance; Saltol; SSR markers.

1. INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important grain crops in Asian countries and it is consumed by two thirds of the world population as their staple food. With the escalating growth rate of world population, the demand for the rice is increasing year by year. Therefore, marginal lands like salt affected lands also need to be utilized to gain maximum production.

Among abiotic stresses, salinity has been recognized as the second most wide spread problem for reduction in growth and productivity of rice in all over the world [1]. Millions of hectares in coastal regions face salinity due to marine/brackish water intrusion to the ground while the inland salinity can occur due to poor irrigation, digestion of ores, human activities etc. In Sri Lanka, approximately 13% of the irrigated lands are affected by salinity stress [2] and this percentage increases gradually in both coastal regions and inlands [3].

Most of the rice varieties are extremely sensitive to salinity during young seedling stage and early development stage [4]. Even though there are agronomical practices like improved field drainage, maintenance of adequate amount of water around 2 to 3 cm height until early reproductive stage [5], establishment of crop by transplanting [3] and use of organic manure instead of inorganic fertilizer are used to address salinity, these practices are not cost effective, efficient and favorable for a long-term solution. Hence, cultivation of durable resistant rice varieties appears more appropriate as a long lasting solution. Therefore, identification of salt tolerant varieties and their transformation into high yielding cultivars is necessary in order to utilize salt affected lands for sustainable rice cultivation.

Salinity tolerance is considered as a quantitative trait as it is governed by a collection of many genes that are called quantitative trait loci (QTL) *Saltol* is one such previously reported major QTL which is reported to be responsible for the salinity tolerance for many tolerant rice cultivars [6]. DNA based molecular markers which are used in marker assisted selection (MAS) are extensively used to tag QTL with phenotypic characters to accelerate breeding processes Hence, this study was conducted to evaluate the growth performance of the seedlings of twenty rice cultivars under salt stress and to detect their genotype at *Saltol* region aiming at finding the relationship with salt tolerance in order to use them in marker assisted selection and gene mapping studies.

2. MATERIALS AND METHODS

2.1 Planting Material

The phenotypic evaluation of the germplasm was conducted in a rain sheltered plant house at Wayamba University of Sri Lanka in mid 2013 Twenty rice germplasm from diverse genomic background representing traditional and improved varieties were collected from Plant Genetic Resources Centre, Gannoruwa, Sri Lanka, and Rice Research and Development Institute (RRDI), Batalagoda, Sri Lanka (Table 1)

Table 1. Varietal information and level of salinity tolerance by standard evaluation score (SES) and salinity survival index (SSI)

Variety	Accession No*	Cultivation background	Average of SES	Salinity tolerance	SSI
Kaluheenati 1(KH1)	003989	Traditional	4	T	0.435
Kaluheenati 2(KH2)	011041	Traditional	5	M	0.410
Lanka Samurdhi (LS)	008921	Improved	8.5	S	0.152
Kuruluthuda (KT)	004759	Traditional	6	M	0.354
Suwadel (Suw)	010729	Traditional	9	HS	0.108
Pokkali (Pok)	005556	Traditional	3.5	T	0.525
Maa Wee (Maa)	008551	Traditional	3.5	T	0.585
Al Wee (Al)	004023	Traditional	3	T	0.738
Goda Wee (Goda)	006182	Traditional	2	HT	0.778
Moraberakan (Mor)	006897	Traditional	6	M	0.379
Muhudu Ralla (MR)	004773	Traditional	6	M	0.321
Kivul Handiran (Kivul)	004106	Traditional	4	T	0.454
Rathu Heenati (RH)	004992	Traditional	6	M	0.351
Basmathi (Bas)	006820	Traditional	7	S	0.268
Bw 400*	005311	Improved	3.5	T	0.488
At 401*		Improved	3.5	T	0.638
Bg 357*		Improved	8	S	0.240
Ld 356*		Improved	6	M	0.371
At354*		Improved	2	HT	0.763
Bg352*		Improved	8	S	0.223

Varieties with accession numbers were obtained from Plant Genetic Resources Centre, Gannoruwa Sri Lanka
Varieties were obtained from Rice Research and Development Institute, Bathalegoda Sri Lanka HT- highly tolerant T- tolerant M- moderately tolerant S- susceptible HS- highly susceptible

Table 2. Sequences, map position and annealing temperatures of six SSR markers close to *Saltol* region

SSR marker	Sequence information 5'-3'	Position in the consensus map (Mb) (www.gramene.org)	Annealing temperature ℃
RM10772	GCACACCATGCAAATCAATGC CAGAAACCTCATCTCCACCTTCC	12.16	55
RM1287	GTGAAGAAAGCATGGTAAATG CTCAGCTTGCTTGTGGTTAG	10.83	54
RM10694	TTTCCCTGGTTTCAAGCTTACG AGTACGGTACCTTGATGGTAGAAAGG	10.96	60
RM493	GAGGTGAGTGAGACTTGACAGTGC GCTCATCATCCAACCACAGTCC	12.28	60
RM10864	GAGGTGAGTGAGACTTGACAGTGC GCTCATCATCCAACCACAGTCC	14.25	60

2.2 Extraction of Genomic DNA and PCR Amplification

Genomic DNA was extracted from 14 day old tender plant leaves using a mini-prep method [7] DNA samples were amplified using six SSR markers located between 10-15 Mb, on the *Saltol* QTL region of the chromosome 1 [6,8,9] (above Table 2) Amplification profile consisted of initial denaturation at 95°C for 5 minutes followed by 35 cycles of denaturation for 1 minute at 95°C, annealing at marker specific temperature (Table 2) for 30 seconds, elongation at 72°C for 5 minutes and final extension at 72°C for 5 minutes. The amplified products were electrophoresed on 3% agarose gel and stained with ethidium bromide SSR allelic composition at each of the defined loci was determined for each genotype.

2.3 Establishment of Hydroponic System with Saline Stress

Hydroponics system was established according to the procedure described by Gregorio et al [1] Germinated seeds (two replicates with 10

seedlings per each) from each germplasm with emerging roots around 1 to 2 cm were placed according to the Completely Randomized Design (CRD) on the styrofoam floats without damaging the roots. Transplanted seedlings were allowed to stand in water for 2 days to recover damages during transplantation. After 2 days, Yoshida's nutrient solution was added with an appropriate amount of NaCl (analytical grade) to give 6 dS/m. The salinity was increased up to 12 dS/m by adding NaCl after 24 hours. The pH was adjusted daily and the solutions were renewed every 8th day. Control experiment was maintained with four seedlings under Yoshida's nutrient solution without NaCl by providing the same conditions as that of NaCl treated seedlings.

2.4 Estimation of Growth Performance

The growth parameters of the seedlings such as root length (RL), shoot length (SL) and root fresh weight (RFW) were measured at the 21st day of salinity treatment. Plant materials were oven dried for 3 days at 70℃ and shoot dry weight (SDW) and root dry weight (RDW) were measured. Mean values of all parameters under salinity ($V_M$sal) were converted to relative values (RV) [5] as indicated below,

$$RV = \frac{V_{M\text{Sal}}}{V_{M\text{Con}}}$$

where V is the respective value of recorded parameters such as root length, shoot length etc., $_M$sal is the mean value under salinity and $_M$con is the mean value under control.

2.5 Assessment of Germplasm by Modified Standard Evaluation Score of Visual Salt Injury (SES)

The degree of visual injures showed by the seedlings on 16th and 21st day were evaluated using the standard evaluation score method described by Gregorio et al. [1]. According to the morphological appearance, SES was assigned to each variety from 1 to 9 which is represented by 1: Highly Tolerant, 3: Tolerant, 5: Moderately Tolerant, 7: Susceptible and 9: Highly Susceptible to the salinity and, the mean value of SES was calculated.

2.6 Assessment of Germplasms by Salinity Survival Index (SSI)

The number of dead plants was counted on every 3rd day after salinization and the respective seedling survival percentage of each day was calculated until 21 days after salinization (DAS). The SSI was calculated using the formula,

$$SI = \sum_{k=1}^{n} D_k S_k \Big/ \left(\sum_{k=1}^{n} D_k\right) 100$$

where, D is the Day After salinization (DAS), S is the survival percentage of that particular day, n is the total period in DAS (in this experiment n is 21 DAS), D_k is the DAS at kth data collection, S_k is the survival percentage of kth data collection and k=1, 2, 3....n. as reported by Wijerathna et al. [7].

2.7 Statistical Analysis

Relative values of each parameter, SSI, root length, shoot length, root fresh weight, root dry weight and shoot dry weight were analyzed by analysis of variance (ANOVA) using SAS version 9.1.3 [10]. Cluster analysis was done using SSR markers based on Jaccard's similarity coefficient following the unweighted pair group method with arithmetic mean (UPGMA) by SPSS 16.0 version [11]. Alleles obtained from SSR markers were analyzed using PowerMarker 3.25 version [12].

3. RESULTS AND DISCUSSION

3.1 Assessment of Salinity Tolerance by Standard Evaluation Score (SES)

The modified standard evaluation score method recommended by International Rice Research Institute, Philippines, was used in rating the visual symptoms of seedlings caused by salt stress. Selected varieties of this study demonstrated all five categories of SES, after 21 days of salinization. Accordingly, the traditional Suwandel variety was highly susceptible to the salinity indicating 9 in SES. Next highest tissue damages were observed in Lanka Samurdhi, Bg357 and Bg352 indicating the SES levels of 8.5 to 8. The minimum visual injuries or the highest tolerance ability was demonstrated in Goda Wee and At354 with SES value of 2 (Table 1).

3.2 Assessment of Salinity Tolerance by Salinity Survival Index (SSI)

It is important to assess survival potential of germplasms under salt stress with a quantitative parameter as such parameter is convenient for the analysis, mapping studies and it is independent of personal skills. A quantitative parameter called salinity survival index was

measured giving maximum weight for the plants that survived throughout whole period while minimum weight was given for the plants that died at earliest possible. In this study, SSI distributed within the range of 0.108 to 0.776 among twenty varieties. The highest SSI was recorded from Goda Wee (0.776), a traditional rice variety, followed by At354 (0.763), a high yielding improved rice variety, and Al Wee (0.738), another traditional variety. The lowest SSI was exhibited by Suwandel (0.108), Lanka Samurdhi (0.152) and Bg352 (0.223) (Table 1), following the same pattern as SES. Two traditional varieties, Maa Wee and Al Wee and an elite variety, At401 showed more tolerability towards salinity stress than the tested accession of Pokkali. This may probably be due to the fact that the prevalence of different accessions of Pokkali, the well known salt tolerant variety, possessing different alleles [6]. Fig. 1 shows the dropping pattern of the survival percentage against the days after salinization that was plotted for the six varieties. Three varieties, Goda Wee, At354 and Al Wee showed sudden drop in survival only at 18th day while Suwandel, Lanka Samurdhi and Bg352 showed the same drop at the 8th day after salinization.

Standard evaluation score of visual salt injury is the mostly used screening technique for salinity tolerance in rice as reported in previous studies. It was reported that visual symptom rating is adequate to determine the level of tolerance for breeding purposes as it was correlated well with yield performance in saline rice fields [1]. However, in addition to SES, in this study we used a novel parameter, SSI, which is mostly similar to SES, but distributed quantitatively. Although SSI was previously developed for the assessment of salt stress in rice under saline soil [7], the results of this study also showed a vast range of variation among germplasm even with the saline hydroponic system, proving to be useful in both soil and hydroponics designed for screening for breeding purpose and gene mapping studies.

3.3 Assessment of Salinity Tolerance by Relative Growth Parameters

The objective of this assessment was to identify the ability to perform under salt stress condition and to compare the performance among varieties regardless their performance under non-stress condition. Therefore, the parameters measured such as root length, shoot length, root fresh weight, root dry weight and shoot dry weight were analyzed with the relative values. Accordingly, all the relative parameters except relative shoot dry weight were highly significant (Table 3). Also all the parameters were significant under control indicating the prevalence of different genetic variability among germplasm. This genetic variability can be nullified with the relative assessment of each trait. Therefore, the variability of relative values reflects extra genetic potential exhibiting the survival ability under salinity.

With regards to the relative root length, maximum relative root length under stress was obtained by Maa Wee and it has exceeded the root length even than the control. The higher reduction of relative root length was displayed in Suwandel, Basmati, Bg357, Bg352 and Muhudu Rella when compared to the non-stress condition and these performances are compatible with respective SSI and SES. Kuruluthuda, Pokkali, Maa Wee and At401 showed increased relative root length under salinity than under the control. This fact indicates that these varieties have the ability to increase the root biomass under stress condition, probably may be due to a strategy existed to overcome salt stress.

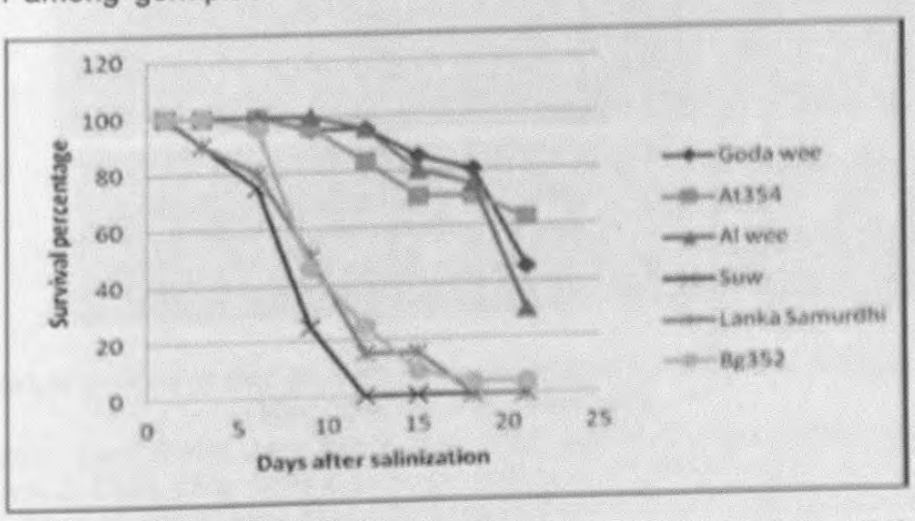

Fig. 1. Survival percentages of six varieties with the days after salinization

With respect to the relative shoot length, Suwandel displayed the maximum reduction and Bw400 displayed the minimum reduction which exceeded even under the control. Also Bw400 exhibited minimum reduction in other traits such as relative root fresh weight, relative shoot dry weight and relative root dry weight under salinity stress. Obviously, under salt stress, reduction of biomass is experienced in most of the crop plants [13]. In this study, we observed different reduction capacities in terms of root biomass and shoot biomass, in twenty rice germplasm under salt stress condition and found that there are some germplasm showing minimum biomass reduction capacity, namely Bw400, At354 and At401, performing better than the tested accession of Pokkali. Bw400, At354 and At401 also were able to categorize as 'Tolerant' or above based on SES. Moreover, Ma Wee, Al Wee and Goda Wee also showed considerably high amount of relative shoot and root biomasses under salt stress compatible to the results of SSI.

3.4 Assessment of Allelic Variation at *Saltol* Region among Germplasms

One objective of this study was to identify SSR markers that are linked with *Saltol* QTL located on chromosome 1 in a diverse germplasm collection. Knowing of the polymorphic markers closer to *Saltol* region would also be useful to select parental lines in developing mapping population and breeding programs. Also polymorphic markers are useful to select background and foreground of known genes to be used in MAS [14,15]. Therefore, twenty rice germplasms were evaluated for the genetic diversity of the region closer to the promising QTL *Saltol* in chromosome 1 using SSR markers. Fig. 2 indicates the gel images showing different banding patterns of amplified DNA. Five markers used were highly polymorphic and 23 alleles were amplified with the band size of 100-300 bp. The highest number of alleles was found in RM 1287 (7), followed by RM493 (5), RM10772, RM10694 (4) and RM 10864 (3) which gave the lowest number of alleles (Table 4). Locus RM 1287 showed the highest polymorphism information content (PIC) value (0.8078) followed by RM493 (0.7470), RM10772 (0.6454). RM10694 (0.6142) and RM10864 (0.4824). The average PIC value was 0.6594 and it ranged from 0.4824 to 0.8078 (Table 4). Therefore, out of 5 markers selected in the 10-15 Mb of *Saltol* region on chromosome 1, RM1287 would be more useful for screening of rice germplasm.

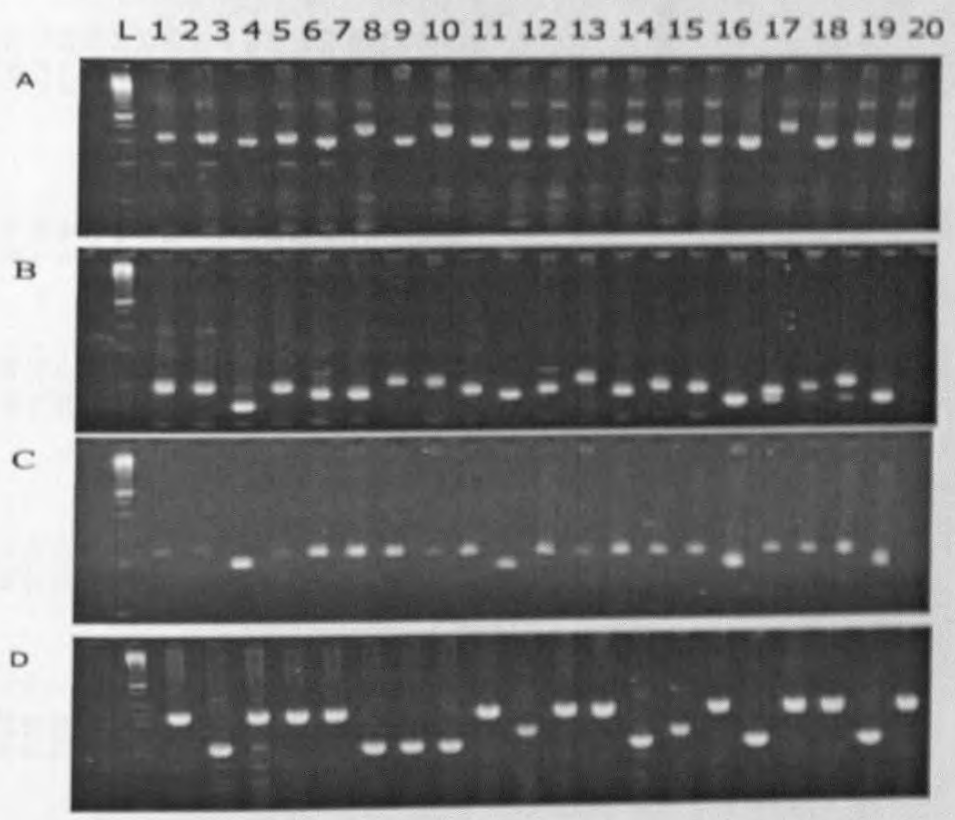

Fig. 2. SSR marker profile of rice germplasms generated by (A) RM10772, (B) RM 1287, (C) RM10694 and (D) RM10864

Lane L: 100 bp ladder, Lane 1: KH1, Lane 2:KH2, Lane 3: LS, Lane 4: KT, Lane 5: Suw, Lane 6: Pok, Lane 7: Maa, Lane 8: Al, Lane 9: Goda, Lane 10: Mor, Lane 11: MR, Lane 12: Kivul, Lane 13: RH, Lane 14: Bas, Lane 15: Bw400, Lane 16: At401, Lane 17:Bg357, Lane 18: Ld356, Lane 19: At354, Lane 20: Bg352

Table 3. Means of the growth performances and relative values measured under salinity stress and control conditions

Variety	RL			SL			RFW			SDW			RDW		
	Stressed (cm)	Control (cm)	Relative	Stressed (cm)	Control (cm)	Relative	Stressed (mg)	Control (mg)	Relative	Stressed (mg)	Control (mg)	Relative	Stressed (mg)	Control (mg)	Relative
KH1	26 11	26 34	0 99	28 82	66 38	0 43	124 31	848 40	0 15	46 78	310 20	0 15	10 73	64 40	0 17
KH2	16 56	19 72	0 84	33 70	65 20	0 52	90 80	459.00	0.20	74 76	241.75	0 31	14 58	44 25	0 33
LS	13 8	16 64	0 83	15 59	43 48	0 36	164 50	450.00	0 37	62 40	107.00	0 58	15 07	35 40	0 43
KT	23 21	22 78	1 02	27 84	60 32	0 46	306 73	529.40	0.58	67 40	188.00	0 36	14 17	33 80	0 42
Suw	9 97	15 4	0.65	16 00	52 75	0 30	171.50	288.00	0.60	65 40	124.00	0 53	10 16	21 00	0 48
Pok	17 43	16 52	1 05	36 94	65.70	0 56	167 39	495 40	0.34	79 91	187.80	0 43	13 54	31 80	0 43
MW	20.85	19.7	1.06	28.20	57.33	0 49	110 34	414.33	0.27	44.40	143.67	0 31	9.79	25 67	0 38
Al	20 19	29 16	0 69	43 63	65 17	0 67	163.91	458.75	0.36	80 62	171.33	0 47	11 74	30 50	0 39
Goda	18.14	23 88	0.76	31.55	56 76	0 56	168 29	609.00	0.28	64.00	187.20	0 34	14 44	37 40	0 39
Mor	18.7	24 96	0.75	22 52	47 86	0 47	81 26	546.40	0.15	41 60	171.00	0 24	8 90	36 40	0 24
MR	10 79	18 94	0.57	26 20	59 34	0 44	100 29	507.20	0.20	53 20	215.80	0.25	9 00	49 00	0 18
KH	18 34	22.58	0.81	40.37	72.30	0 56	223.85	582.60	0.38	91.16	283.50	0.32	13.39	45 20	0 30
RH	16 05	19 18	0 84	36 28	67 85	0 53	194.91	609.73	0.32	88 50	265.00	0 33	46 44	52 50	0 88
Bas	9.63	14.08	0.68	22 00	57 00	0.39	127.90	512.60	0.25	47.12	234.60	0.20	9.12	45 60	0 20
Bw400	14 35	15 75	0.91	28 42	21 25	1 34	234 80	195.50	1.20	104.60	144.00	0 73	18 41	22 50	0 82
At 401	19 66	18 6	1 05	29 46	48 88	0 60	246.78	556.00	0.44	193 72	303.25	0 64	18.56	46 00	0 40
Bg 357	11 62	20.33	0.57	19 63	45 10	0 44	145 85	435.75	0.33	117.10	191.75	0.61	21 53	37 50	0 57
Ld 356	15 06	19 9	0.76	23 10	46 85	0.49	188.74	361.50	0 52	65 00	155.00	0 42	12 22	27 50	0 44
Al354	21 96	24 86	0.88	29.78	46 48	0 64	164 50	188.50	0.87	67 60	119.67	0 56	16 30	20 33	0 80
Bg352	16 25	25 8	0 63	22 54	51 55	0.44	112.00	157.20	0 71	61 95	109.40	0 57	15 87	22 20	0 71

**** Significant at P = 0 001, ** Significant at P = 0 01, ns not significant (Significant level of each parameter was obtained from ANOVA). RL (root length), SL (shoot length) RFW (root fresh weight) SDW (shoot dry weight) and RDW (root dry weight)*

Table 4. Allelic variability of five markers located near *Saltol* region on chromosome 1

Marker	Allele no	Major allele frequency	Gene diversity	PIC
RM10772	4.0000	0.4000	0.7000	0.6454
RM1287	7.0000	0.2500	0.8300	0.8078
RM10694	4.0000	0.4211	0.6759	0.6142
RM493	5.0000	0.3158	0.7812	0.7470
RM10864	3.0000	0.5500	0.5650	0.4824
Mean	4.6000	0.3874	0.7104	0.6594

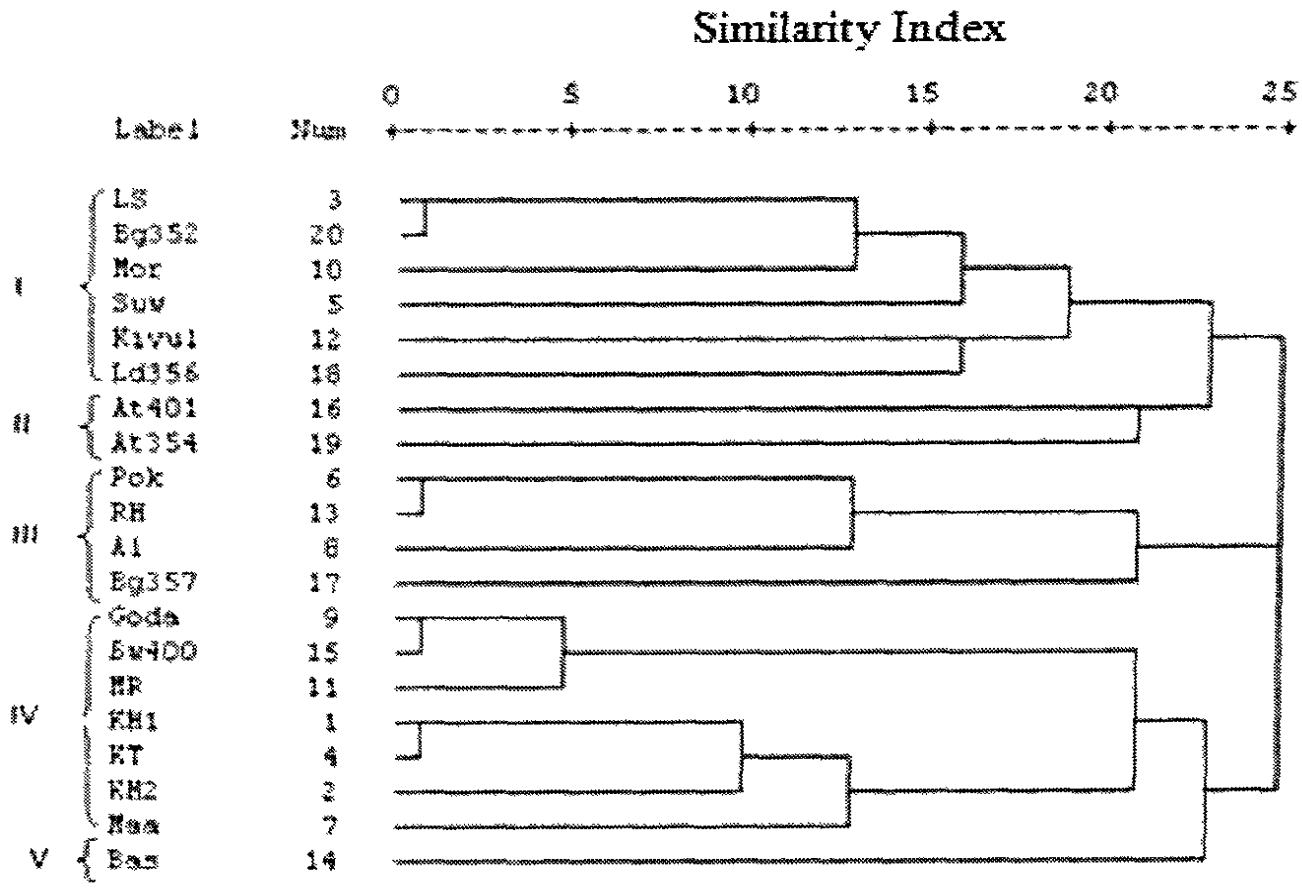

Fig. 3. UPGMA dendrogram using Jaccard's similarity coefficient among twenty rice germplasms based on five SSR markers in the *Saltol* QTL region of chromosome 1

The dendrogram (above Fig. 3) obtained from 23 alleles of 5 SSR primers showed 5 major clusters at similarity levels of 23. More susceptible germplasms like Suwandel, Bg352 and Lanka Samurdhi were grouped in cluster I. Two improved, highly salinity tolerant varieties, At354 and At401 were separately clustered in cluster II. Cluster III contained two sub clusters of which one sub cluster contained susceptible variety Bg357 while other sub cluster represent tolerant and moderately tolerant varieties. The highest tolerant cultivar, Goda Wee, was clustered together with another tolerant cultivar, Bw400, in a sub cluster of major cluster IV suggesting a similar genetic background at the tested region, *Saltol* of chromosome 1. These two varieties exhibited favorable phenotypic traits for salt tolerance although both were originated from two completely different backgrounds. When the main cluster IV is considered, regardless the sub clusters, it can be noted that all the varieties included are either salinity tolerant or moderately tolerant ones. They are four tolerant varieties, Goda Wee, Bw400, Kaluheenati1 and Maa Wee and, three moderately tolerant varieties, namely Muhudu Rella, Kaluheenati2 and Kuruluthuda. In this cluster except Bw400, all other six varieties are traditional varieties suggesting that these varieties might have been originated from the same ancestor grown in Sri Lanka. It was interesting to note that a salinity susceptible imported variety, Basmathi, was separately clustered in cluster V.

Diversity analysis based on SSR marker alleles linked with *Saltol* clustered the salinity tolerant

and salinity susceptible germplasms as compatible with the distribution pattern of salinity survival index. Comparison of the SSR allele results revealed interesting relationships related to the overall level of salinity tolerance in germplasms and their origin. Based on these alleles, salinity susceptible varieties and tolerant varieties were able to be separately sub-clustered in some main clusters of the dendrogram, which appears to be useful in selecting parental lines for breeding on salt tolerance.

4. CONCLUSION

In this study, of twenty cultivars, two traditional varieties, Goda Wee and Al Wee and, an improved variety, At354 were highly tolerant to the salt level of 12 dS/m, showing more than 0.7 of salinity survival index. Also traditional varieties, Pokkali, Ma Wee, Kivul Handiran and Kaluheenati1 and improved varieties, Bw400 and At401, were categorized as tolerant varieties. All these nine germplasms showed minimum reduction capacities of root and shoot biomass under salinity stress of 12 dS/m confirming their comparative salt tolerant nature. Five markers selected in the 10-15 Mb of *Saltol* region on chromosome 1 were highly polymorphic and of them, RM1287 would be more useful for screening of rice germplasm for salinity tolerance.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Annexure 05

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EXPLORATION OF RELATIONSHIP BETWEEN FRAGRANT GENE AND GROWTH PERFORMANCES OF FRAGRANT RICE (*Oryza sativa* L.) SEEDLINGS UNDER SALINITY STRESS

Wijerathna Y M A M[1], Kottearachchi N S[1*], Gimhani D R[1] and Sirisena D N[2]

epartment of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, Makandura, Gonawila (NWP), Sri Lanka.
oil Science Division, Rice Research and Development Institute, Batalagoda, Ibbagamuwa, Sri Lanka

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Salinity survival index

ABSTRACT

Fragrant rice is associated with poor agronomic traits and low tolerability to salt stress. Present study was conducted to examine the relationship between fragrant intensity of Sri Lankan rice germplasms and their seedling growth under salt stress. It was reported that correct copy of *badh2* gene is associated with more salt tolerance than the mutated version of *badh2* gene. Fifteen accessions that exhibited different levels of fragrance were selected and they were categorized into four fragrant intensity groups. Rice seedlings were grown under three salinity levels, 0.73 dSm^{-1} (control), 4 dSm^{-1} and 8 dSm^{-1} and their survival percentage, root length and shoot length were measured. A novel formula of Salinity Survival Index (SSI) was derived from survival percentages and analysis of variance revealed that survival index under salinity stress was significantly associated with the fragrant intensity groups of rice. As the fragrant intensity increases, SSI, root length and shoot length decreases. A fragrant accession that were exceptionally salt tolerant were also disclosed. Most of the Sri Lankan fragrant rice germplasms do not possess the predominant *badh2.1* fragrant allele. These findings would be useful in rice improvement breeding programs designed for fragrance and salt tolerance.

Corresponding author

mail: kottearachchins@yahoo.com (Kottearachchi)

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1 Introduction

Fragrant trait of *Oryza sativa* L. constantly adds value to the quality of rice attracting consumer preference. Demand for high quality aromatic rice is increasing day by day with the living standard of people. Basmati and Jasmine rice are the most popular two aromatic rice varieties in the world. 2-Acetyl-1-Pyrroline (2-AP) is the major compound responsible for the fragrance in Basmati and Jasmine style rice (Buttery et al., 1983; Lorieux et al., 1996; Widjaja et al., 1996; Yoshihashi, 2002). However, there are other traditional aromatic rice varieties which exhibit different volatile compounds other than 2AP such as acetaldehyde, propanol, 2-butanone, pentanal, hexanol etc. (Tsugita, 1986; Widjaja et al., 1996; Singh et al., 2007; Yang et al., 2008) and hence, fragrant intensity of a variety may be associated with presence of multiple volatile compounds produced by different biochemical pathways

Bradbury et al. (2005) reported that an 8 bp deletion in the 7th exon of *badh2* gene, referred as fragrant gene, located on 8th chromosome is responsible for 2AP production. The wild type *badh2* gene encodes the betaine aldehyde dehydrogenase enzyme (BADH2) which is reported to be associated with salt tolerance in rice (Niu et al., 2008; Fitzgerald et al., 2010). Recently, Kovac et al. (2009) revealed other mutations that cause BADH2 enzyme non functional and these mutations scattered from 1st exon to 14th exon of *badh2*, were named as *badh2.1, badh2.2, badh2.3, badh2.4 and badh2.5, badh2.6, badh2.7, badh2.8, badh9* and *badh10*. It s reported that *badh2.2*, is associated with fragrance of some germplasm in China (Shi et al., 2008). However, most of the popular cultivars including Basmati and Jasmine rice contain predominant *badh2.1* allele

Rice is salt sensitive crop and sensitivity of rice to salinity stress varies with the growth stage. In general, more sensitivity was reported at the young seedling stage of rice and at the stage of reproduction (Flowers & Yeo,1981; Lutts et al., 1995). Heenan et al. (1988) have also reported that most of rice varieties are extremely sensitive to salinity during young seedling stage and early developmental stages. Hence, improvement of salt tolerance in rice should be targeted at these growth stages that are more sensitive to salinity stress since it can significantly affect grain yield.

Basmati is the usual donor of the fragrant gene in the rice breeding programs although it shows the poor combining ability with some high yielding varieties (Bourgis et al., 2008). It has been reported that aromatic rice are more susceptible to saline soil than non fragrant rice (Niu et al., 2008; Fitzgerald et al., 2010) Therefore, it is necessary to evaluate growth performances of Sri Lankan traditional aromatic rice as many of them are commercially unexploited and they have the potential to be used in quality improvement breeding programs instead of Basmati However, there are only few reports on high-quality aromatic rice linked to salt stress. Therefore, this study was conducted to examine the relationship between fragrant intensity and seedling growth of Sri Lankan rice germplasms under salt stress and to detect the fragrant allele at *badh2* locus.

2 Materials and methods

2.1 Plant materials

Seeds of 80 rice accessions were obtained from the Plant Genetic Resources Center (PGRC), Sri Lanka and Rice Research and Development Institute (RRDI), Batalagoda, Sri Lanka. Fifteen accessions were selected for this study out of 80 accessions of which the fragrance was evaluated by KOH method (Sood & Siddiq, 1978) The 15 accessions were comprised of 2 high yielding varieties i.e. At401 and At405 that were derived from same parental lines but possessed different fragrant traits, Pokkali, the well known salt tolerant variety, 2 Basmati varieties and 10 traditional accessions that are popular among commercial growers in Sri Lanka

2.2 Fragrance evaluation

Aroma in leaves was determined according to method described by Sood & Siddiq (1978) by mixing 10 ml of 0.5% potassium hydroxide (KOH) solution with the chopped leaf sample obtained from rice plants at tillering stage. Basmati217 was used as the reference for the sensory aroma evaluation and the smell of Basmati217 was categorized as highly fragrant The KOH treated leaf samples were smelled and fragrant intensity score (FIS) was assigned ranging from 0 to 3 which is indicated by 0: Non-fragrant, 1: fragrant, 2. Highly fragrant and 3: Superior fragrant according to the procedure described by Amarawathi et al (2008) by a sensory evaluation panel of five members.

2.3 Detection of badh2 alleles

DNA was extracted from each accession and subjected to PCR amplification with the mixture consisted of 1.5 µl of 10X buffer, 1.2 µl of (2.5 mM) dNTPs, 1 µl (20 pmol/µl) of primers (**ESP**:TTGTTGGAGCTTGCTGATG, **IFAP**:CATAGGAGCAGCTGAAATATATACC, **INSP**:CTGGTAAAAAGATTATGGCTTCA, **EAP**:AGTGCTTTACAAGTCCCGC; Bradbury et al., 2005) and 0.2 µl of (5 units/µl) Taq DNA Polymerase in total volume of 15 µl. Amplified PCR products were electrophoresesed by 1.3% agarose gel followed by ethidium bromide staining.

2.4 Preparation of saline soil and establishment of seedlings

Saline soil was prepared based on the procedure described by Sirisena & Hemachandra (2007) with slight modifications The untreated soil contained +75.2% of clay, 18.6% of silt and 6.2% of sand and the pH was 8.1 while the Electric Conductivity of Saturated Extract (ECe) of the soil was 0.73 dSm^{-1} The calibration graph was plotted in order to determine

the correct amount of NaCl to be added and accordingly, 0.1 g, 0.2 g, 0.3 g, 0.4 g, 0.5 g and 0.6 g of NaCl were added separately to 250 g soil samples with distilled water up to saturated level and samples were stirred over night ECe of each sample was measured at room temperature (30°C) after extracting water by a vacuum pump and the graph was plotted on between ECe and added weight of NaCl per 250 g of soil. Required amount of NaCl to maintain 4 dSm^{-1} and 8 dSm^{-1} was calculated by the graph. Plastic trays (42 cm x 32 cm x 6 cm sized) were filled with 5 kg of soil and three levels of ECe with 0.73 dSm^{-1} (control), 4 dSm^{-1} and 8 dSm^{-1} were established by saturating the soil with artificially prepared saline water added with estimated NaCl. Electrical conductivity (EC) of prepared soil treatments also measured by inserting the portable EC meter- probe into the saturated soil and another graph was plotted between the ECe and EC to maintain the same ECe throughout the experimental period. EC of saturated soil was monitored and de-ionized water was added if necessary to the soil tray to maintain correct EC. Each salinity treatment was replicated two times.

Seeds were soaked and after emerging radicals, five day old seedlings were transferred into trays prepared with three salinity levels, 0.73 dSm^{-1}, 4 dSm^{-1} and 8 dSm^{-1}. Seeds of each genotype were planted in a line keeping about 2 cm gap between plants comprising of 20 seedlings per accession.

2.5 Evaluation of seedling survival

Seedling survival percentage of each accession was calculated once every three days after transplanting (DAT) till 21 days of the experiment. The results of seedling survival percentages were expressed as a weighted Salinity Survival Index [SSI] to convert them into quantitative data. This index provides maximum weight to the seedlings that survive throughout the 21 DAT, and is calculated from the formula as follows; SSI = (3xS1+6xS2+. . .21xS7)/ (3+6+.... 21) 100, where S1, S2...S7 are the survival percentages that had observed on 3rd, 6th, 21st DAT. The generalized form of the formula was derived as –

$$SSI = \sum_{k=1}^{n} D_k S_k \Big/ \left(\sum_{k=1}^{n} D_k\right) 100$$

where, D is the DAT, S is the survival percentage, n is the total period in DAT (in this experiment n is 21 DAT), Dk is the DAT at kth data collection, Sk is the survival percentage of kth data collection and k=1,2,3 n If the assessment is done in hydroponics, data can be collected after salinization, not DAT, because transplantation is not required in hydroponics The maximum index is 1.0 if all seedlings survived throughout the whole period, while the minimum index is 0 if all seedlings died on the first day of data taken.

2.6 Evaluation of salt injury

Visual salt injury at seedling stage was assessed by the standard evaluation score (SES) for each selected accession and it was from 1 to 9 rating which is indicated by 1: Highly Tolerant, 3: Tolerant, 5. Moderately Tolerant, 7 Susceptible and 9: Highly Susceptible as described by Gregorio et al (1997) for salt tolerance on the basis of the morphological appearance of seedling growth after 21 days of salinity treatment.

2.7 Evaluation of seedling growth

After 21 days of the salt stress, remaining seedlings were taken from soil media without damaging the roots. Shoot length and root length of seedlings of each accession was measured Mean shoot length and mean root length were calculated and were analyzed to study the seedling growth performances under salinity stress over different FIS.

2.8 Statistical analysis

Three salinity treatments of 0.73 dSm^{-1}, 4 dSm^{-1} and 8 dSm^{-1} and four types of FIS, 0, 1, 2 and 3 were followed in a factorial experiment with completely randomized design. Mean SSI, mean root length and mean shoot length were analyzed by analysis of variance (ANOVA) using Minitab version 15 The mean SSI over FIS was analyzed by regression model after creating indicator variables for fragrant categories by Minitab version 15.

3 Results and Discussion

3.1 Evaluation of fragrance and detection of badh2 alleles

Table 1 represents the result of fragrant intensity of leaves, these results revealed that the strengths of the aroma were different among accessions. Out of 80 selected accessions (Data not shown) the traditional variety Kuruluwee gave the highest fragrant intensity and it was higher than the reference variety Basmati217. The results of the present study revealed the presence of several fragrant and highly fragrant accessions as compared to the Basmati217. Of all 80 accessions, only two local accessions, At 405 and Suwanda-samba exhibited the presence of *badh2.1* allele. None of the Sri Lankan traditional fragrant accessions revealed the *badh2.1* allele at 7 th exon region and instead wild type allele was detected at the locus

3.2 Evaluation of seedlings survival

In this study, in order to detect the relationship between fragrant intensities and seedling growth under salt stress, survival percentages of different accessions under salt stress were examined (Figure. 1). Salinity Survival index (SSI) was calculated using survival percentages in order to convert the data into a quantitative parameter Analysis of variance revealed that SSI is significantly ($P < 0.001$) associated with different level of fragrant scores (Table 1) The regression analysis was conducted separately for 4 dS/m and 8 dS/m salinity levels and the regression models were obtained as follows by using FIS as indicator variables

a) SSI at 4ECe = 0.463 + 0.495 (FIS_0) + 0.287 (FIS_1) + 0.360 (FIS_2)
b) SSI at 8ECe= 0.111 + 0.652 (FIS_0) + 0.487 (FIS_1)+ 0.406 (FIS_2)

According to the coefficient obtained at 8ECe (Model: b), non-fragrant rice (FIS_0) has an average of 0.652 higher SSI than superior fragrant rice (FIS_3) while fragrant rice (FIS_1) has average of 0.488 higher SSI than superior fragrant rice. The average difference between highly fragrant rice (FIS_2) and superior fragrant rice (FIS_3) was 0.406 at the ECe level of 8 dSm^{-1}. In general, accessions with higher fragrant scores showed the higher reduction in SSI when the salinity stress was increased from 4 dSm^{-1} to 8 dSm^{-1}.

Non-fragrant accessions, At 401 and Pokkali, exhibited the highest SI in both 4$dSm^{1}$to 8 dSm^{-1} levels. Although Kaluheenati (Acc. No. 003989) was a fragrant accession it demonstrated highest SSI as that of Pokkali in both 4 dSm^{-1} to 8 dSm^{-1} levels. Least SSI was observed in Kuruluwee (Acc. No. 004903) which was the one and only superior fragrant accession detected out of 80 accessions. Basmati217 (Acc. No. 007018) also gave very low SSI at 8ECe level. The results showed that the trend of the SSI decreases as the fragrant intensity increases.

3.3 Evaluation of seedlings salt injury

The genotypes classified into five groups from highly tolerant (SES: 1) to highly susceptible (SES: 9) according to visual salt injury observations are presented in Table 1. None of the non-fragrant genotypes were ranked as susceptible or highly susceptible. All highly fragrant or superior fragrant genotypes exhibited either susceptible or highly susceptible phenotype. Kaluheenati showed the exceptional tolerance even at 8 dSm^{-1} as that of salt tolerant check variety, Pokkali.

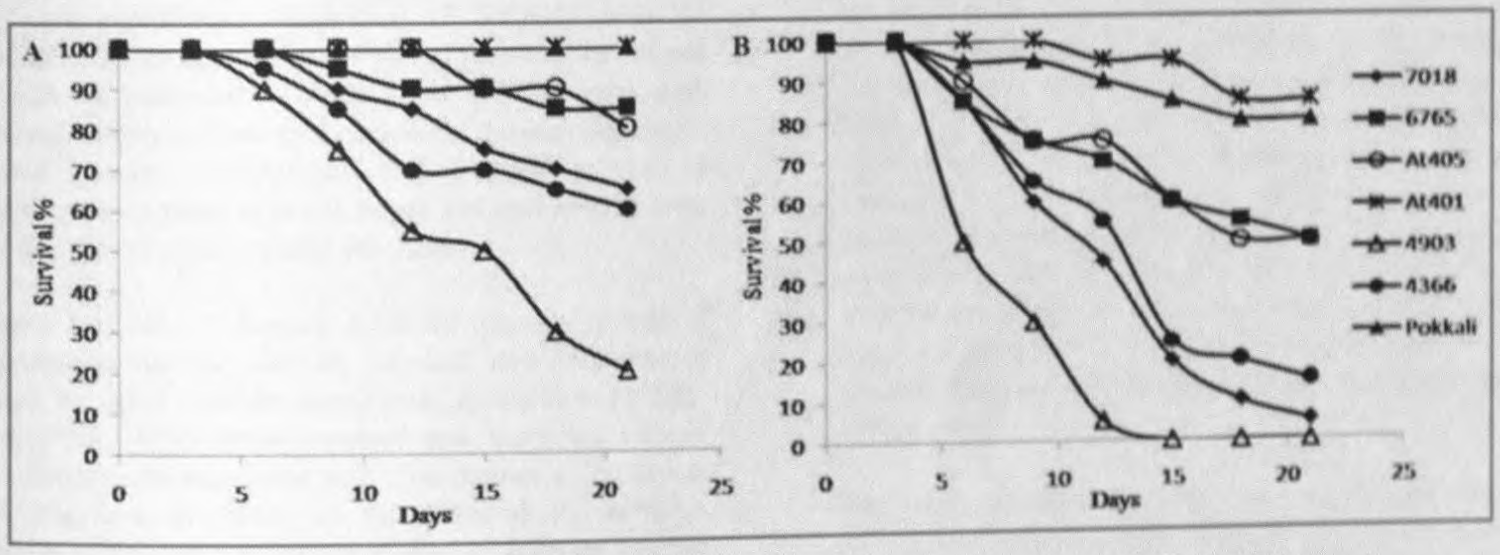

Figure 1 Survival percentages of different accessions under salt stress; A: 4ECe, B: 8ECe.

Table 1 Fragrant intensity score (FIS), alleles of *badh2*, standard evaluation score (SES) and salinity survival index (SSI) of tested accessions.

Accession No.	Accession name	FIS	Allele at 7th exon	SES of visual salt injury		SSI	
				4Ec	8Ec	4Ec	8Ec
**	At 401	0	wild	1	1	1.000000	0.924286
**	Pokkali	0	wild	1	1	1.000000	0.857143
003521	Hondarawal	0	wild	1	5	0.896429	0.719643
006775	Basmati-fine	0	wild	1	5	0.892857	0.626786
011041	Kaluheenati	1	wild	3	3	1.000000	0.683929
003989	Kaluheenati	1	wild	3	3	1.000000	0.905357
003507	Suwanda-samba	1	*badh2.1*	3	5	0.928571	0.700000
003867	Hondarawala	1	wild	3	5	0.630357	0.532143
010729	Suwadal	1	wild	3	9	0.480357	0.480357
04366	Suwanda -Al	1	wild	5	9	0.708929	0.369643
010646	Suwadal	2	wild	5	9	0.692857	0.635714
008921	At 405	2	*badh2.1*	3	7	0.910714	0.626786
004759	Kuruluthuda	2	wild	3	7	0.916071	0.510714
007018	Basmati217	2	*badh2.1*	5	9	0.771429	0.294643
004903	Kuruluwee	3	wild	7	9	0.462500	0.110714

** Obtained from RRDI

Table 2 Means of salinity survival index, shoot length and root length of fragrant rice under salt stress.

Fragrant score / Salt level (ECe)	Non-fragrant	fragrant	Highly fragrant	Superior fragrant
Mean salinity survival index				
4	0.9580[a]	0.7870[b]	0.8134[b]	0.6150[c]
8	0.76[a]	0.5975[b]	0.517[b]	0.1105[c]
Mean shoot length (cm)				
4	18.3[a]	18.96[a]	16.27[b]	10.85[c]
8	13.78[a]	12.52[a]	11.81[a]	6.44[b]
Mean root length (cm)				
4	4.09[a]	3.06[a]	3.58[a]	4.03[a]
8	3.42[a]	2.23[b]	2.56[b]	1.63[c]

Means followed by different letters (a, b and c) within each row show significant difference at $P < 0.05$.

3.4 Evaluation of seedlings by shoot length and root length

Analysis of variance revealed that both shoot length and root length were significantly associated ($P < 0.001$) with the fragrant intensity groups of rice. Means of the shoot length and root length are presented in Table 2. At 8 dSm^{-1} level, both mean shoot length and mean root length showed significant difference between non-fragrant and fragrant groups. In general decreasing trend of shoot length and root lengths were observed as the fragrant intensity increases.

This study was able to identify different intensity groups of rice, probably may be due to the fact that fragrance is generated by other volatile compounds in addition to 2AP. Widjaja et al. (1996) have reported that there are various volatile compounds associated with rice fragrance and hence, overall fragrance is likely to be rendered by complex interactions of volatile compounds leading to different fragrant intensities.

The *badh2.1* allele was detected only in two local rice varieties out of 80 accessions and one of them, At405, has been bred from Basmati parental line. This confirms that *badh2.1* allele is not a predominant allele in Sri Lankan rice gene pool. The association between salt tolerance and fragrant phenotype was previously reported by Niu et al. (2008) and Fitzgerald et al. (2010).

Accordingly, the reason for the low tolerance to salinity has been explained by the loss of the function of BADH2 enzyme in fragrant rice. The role of BADH2 is the metabolism of gamma aminobutyraldehyde which is supposed to play a role in assisting abiotic stress tolerance in plants. Therefore, it was proposed that the absence of functional BADH2 leads to increased levels of 2AP and decreased level of GABA which ultimately lead to loss of salt tolerance (Fitzgerald et al., 2010).

Our data clearly show that when the fragrance increases susceptibility to salt also increases. As the intact *badh2* gene perform a role in salt tolerance, mutated *badh2* gene that causes rice fragrance due to loss of it's function, might be responsible for the susceptibility to salt stress. However, in this study only three aromatic accessions appeared possessing mutated *badh2.1* at 7th exon. . Kovac et al. (2009) reported that there are other alleles of *badh2* such as *badh2.2*, *badh2.3* etc. in other locations of *badh2* gene causing the fragrance in rice. Non-detection of fragrant allele in all fragrant rice accessions used in this study does not mean that fragrance of those accessions was not caused by mutated *badh2* gene. There might be a mutation elsewhere in the *badh2* gene. Important fact to be noted is that SSI decreases as the fragrant intensity increases. The similar trend of increasing salt susceptibility was observed with increasing fragrant intensity, when other parameters, SES, shoot length and root length were examined. Therefore, it is evident that Sri Lankan fragrant rice varieties have an association with the salt susceptibility at seedling stage.

Previously, germination index was used by the Khan et al. (1997) to measure the germination ability of rice seeds under saline condition. Germination index does not reveal the ability to survive of young seedlings after germination. In this study, we derived the SSI to measure the ability to survive under salinity stress for the first time based on the survival % of each accession. SSI was created by giving the maximum weight to the plants that survived until the last day of the data taken and minimum weight was given to the plants that survived till only the first day of data taken. As the index distributed quantitatively, the variation of survivals among accessions were easily observed and analyzed.

In this study Kuruluwee was identified as a superior fragrant variety and thus, the particular variety is worth to be used in fragrance improving breeding programs. Kaluheenati showed highest SSI as that of Pokkali, the salt tolerant check variety and therefore, further experiments are necessary to investigate Kaluheenati as a novel source of salt tolerance in gene mapping studies.

Acknowledgements

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were greatly acknowledged. The authors are thankful to Dr. N.R. Abeynayake for the assistance given on statistical analysis.

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Annexure 06

MICROSATELLITE MARKER BASED HYBRIDITY ASSESSMENT FOR SALINITY TOLERANCE IN RICE

Short Communication

D. R. Gimhani[1], N. S. Kottearachchi[1] and W. L. G. Samarasinghe[2]

ABSTRACT

Development salinity tolerant rice varieties have been a prioritized research area in rice breeding programs in Sri Lanka. Present study was initiated with the cross of At354, a salt tolerant parent and Bg352, a salt susceptible parent aiming at identifying salinity tolerant quantitative trait loci (QTL) in elite rice background. In rice, as getting true hybrids is a technical problem, F_1 progeny was assessed using microsatellite markers that are close proximity to Saltol QTL in chromosome 1. SSR Markers such as RM1287, RM493, RM10772, RM10852 and RM140 were polymorphic between parents. Six true hybrids were selected by polymorphic markers and subsequent generations were developed successfully by single seed descent method. This study explains the feasibility of utilizing microsatellite markers for verification of hybridity, surveying of polymorphic markers in chromosome 1 of At354 and Bg352 and progress towards Recombinant Inbred Line population development.

Keywords: *Hybridity assessment, Microsatellite markers, Rice, Salinity tolerance*

INTRODUCTION

Salinity is the second most widespread soil problem in rice growing countries next to drought (Gregorio *et al.,* 1997). The productivity of most paddy growing lands in Sri Lanka also declines every year due to soil salinity, which arises due to the introgression of seawater during high tide and the rising of shallow saline ground water particularly during the dry season. This condition has aggregated by the recent tsunami disaster experienced by Sri Lanka by leaving large extent of the croplands in coastal area without cultivation due to considerable increment of saline condition in soil. Therefore, development of salt tolerant varieties has been considered as one of the strategies to increase rice production in saline prone areas.

Salinity tolerance in rice is a quantitative trait which is governed by many genes (Islam, *et al.*, 2011). Present study was initiated with the aim of identifying salinity tolerant quantitative trait loci (QTLs) in elite rice background under Sri Lankan environment. In view of this, development of mapping population for salt tolerance is a prerequisite because mapping populations provide an efficient means for identifying genes and studying their associations with various plant traits.

Generally in self-pollinating species, mapping population is generated by crossing homozygous parents which are distinctly different for the trait that is going to be assessed (Collard *et al.,* 2005). However, in rice, getting true hybrids is a technical problem as self- fertilization may occur before out crossing preventing the transfer of desired traits in progenies. In addition, conventionally, the genetic purity of the rice hybrids is assessed by the Grow-Out Test based on specific morphological traits which is time consuming, restricted to a few characteristics, influenced by environmental condition, and inefficient. Consequently selected plants based on such traits may not be true hybrids and such inaccurate identification can adversely

[1] *Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba Univesity of Sri Lanka, Makandura, Gonawila (60170) (NWP), Sri Lanka.*

[2] *Rice Research and Development Institute, Batalagoda, Ibbagamuwa, Sri Lanka.*

affect all stages of future breeding program (Tamilkumar *et al.,* 2009). Therefore, present study reports the use of microsatellite markers for the precise assessment of hybridity in F_1 generation of At354 and Bg352 cross which is then subsequently advanced.

MATERIALS AND METHODS

Experimental site and Plant material

The study was conducted at the Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, Makandura, Gonawila in collaboration with the Rice Research and Development Institute (RRDI), Batalagoda and, the crosses were initiated in 2010. At354, derived from Pokkali and Bg94-1, was selected as the salt tolerant parent and Bg352, derived from Bg380/Bg367-4 cross, was selected as the salinity susceptible parent.

Hybridization and development of mapping population

F_1 seeds produced from At354 and Bg352 cross, treated with the fungicide were established in the nursery. Well grown F_1 seedlings were selected and they were assessed by microsatellite markers that were polymorphic between parents. Selected true F_1 hybrids were advanced to subsequent generations through inbreeding towards recombinant inbred line population (RILs) by single seed descent method (SSD).

DNA extraction

Genomic DNA was extracted from putative F_1 individuals and two parents according to the method described in http://rgp.dna.affrc.go.jp/rgp/protocols/QTL.pdf. The leaf pieces were homogenized with 300 µl of DNA extraction buffer (1 M KCl, 1 M Tris HCl, 0.5 M EDTA). Homogenized mixtures were incubated at 70°C for 20 min. Extracts were centrifuged at 13,000 rpm for 15 min under room temperature. 100 µl of ice cold iso-propanol was added into new eppendorf tubes, and the supernatant of the above centrifuged samples were transferred into them. Solutions were mixed gently. After mixing, tubes were kept at 4°C for 15-30 min and centrifuged at 13,000 rpm for 15 min under room temperature. DNA pellets were washed with 150 µl of 70 % ice cold ethanol by centrifuging at 13,000 rpm for 10 min. Supernatants were removed and pellets were air dried and dissolved in 200 µl of 1/10 TE buffer (10 mM Tris, 1 mM EDTA).

Microsatellite marker analysis

According to the Thomson, *et al.,* 2010, microsatellite markers located at the Saltol QTL in chromosome 1, were selected for the study (Table 01). The primer sequence information was obtained from the published sequence database (www.gramene.org).

PCR was conducted using 8 microsatellite markers with the 15 µl PCR mixture consisted of 5 µl of genomic DNA, 1.5 µl of 10X PCR buffer, 1.2 µl of dNTPs (2.5 mM of each dNTP), 0.8 µl of primer mixture (20 µM of each Forward and Reverse primer) (Integrated DNA Technologies, USA) and 0.25 µl of Taq DNA polymerase (5U/ µl, Sigma, USA). Final volume of the mixture was adjusted to 15 µl by adding sterile distilled water. PCR amplification was performed using thermal cycler, BIORAD (USA) and amplification profile consisted of initial denaturing at 95°C for 5 minutes followed by 35 cycles of 1 minute at 95°C, 30 seconds at relevant annealing temperature (Table 01), 1 minute at 72°C and final extension cycle of 5 minutes at 72°C. Amplified PCR products were analyzed using 3% agarose gel.

RESULTS AND DISCUSSION

F_2, backcross, and RILs are the three primary types of mapping populations used for molecular mapping (Collard *et al.,* 2005). Unlike F_2 or backcross populations RILs produce homozygous or 'true-breeding' lines

that can be multiplied and reproduce without genetic change. In this attempt of producing RILs, as the parents of the cross, At354 and Bg352 exhibited comparatively similar visual appearance and both were in the category of 3 ½ months maturity time, molecular markers were used for screening of segregants. Similar studies have been conducted for the identification of rice hybrids and their respective parents, assessment of plant to plant variation within parental lines and testing the genetic purity of rice hybrids using molecular markers (Yashitola *et al.*, 2002; Nandakumar *et al.*, 2004; Yun *et al.*, 2005; Sundaram *et al.*, 2007).

According to the results of the hybridization, 119 F_1 seeds resulted and only 14 F_1 seeds successfully produced F_1 seedlings exhibiting 11.7% of comparatively low seed viability. Five microsatellite markers of eight, viz., RM1287, RM493, RM10772, RM10852 and RM140 exhibited polymorphism between parents (Figure 01 and 02). These polymorphic markers were used to examine the hybridity of 14 well grown F_1 individuals. Accordingly, hybridity of 6 F_1 individuals was confirmed proving their heterozygosity at the respective loci representing two specific alleles of both parents (Figure 02). Rest of the 8 F_1 individuals were confirmed as off-types as they exhibited only one of the alleles of parents.

By selfing of selected 6 true hybrids, 350 F_2 segregants were raised successfully followed by advancing of subsequent generations by SSD method as illustrated by Figure 03. Assessment of homozygosity of F_5 population indicated that all tested individuals (96 seedlings) were 100 % homozygous which would be tally with the expected level of 92% homozygosity in Mendelian genetics.

CONCLUSION

In present study, hybridity of 6 F_1 individuals derived from AT354/Bg352 cross was confirmed precisely with the RM1287, RM493, RM10772, RM10852 and RM140 microsatellite markers. By selfing of 6 true hybrids, 350 F_2 segregants were raised successfully followed by advancing of subsequent generations to F_5 with the expected level of homozygosity. As the SSR markers used in this assessment are located in close proximity to Saltol QTL, they would possibly be useful for the identification of Saltol QTL in At354 and Bg352 cross.

Table 01: Selected rice microsatellite markers located at chromosome 1.

Locus name	Primer sequence	SSR motif	Annealing T. (C^o)
RM562	CACAACCCACAAACAGCAAG CTTCCCCCAAAGTTTTAGCC	$(AAG)_{13}$	53
RM493*	TAGCTCCAACAGGATCGACC GTACGTAAACGCGGAAGGTG	$(CTT)_9$	56
RM8115	TATATAGTAAATTTGTTTGGTGTAGG ACAGATGGATATTATAAGAAGTAACA	$(TA)_{18}$	50
RM6711	TAGTGATAGGGGTGGTGTGG TTACAAGCATGGGAGTTGGG	$(TAT)_8$	56
RM1287*	GTGAAGAAAGCATGGTAAATG CTCAGCTTGCTTGTGGTTAG	$(AG)_{17}$	53
RM10772*	GCACACCATGCAAATCAATGC CAGAAACCTCATCTCCACCTTCC	$(CTT)_{16}$	55
RM10852*	GAATTTCTAGGCCATGAGAGC AACGGAGGGAGTATATGTTAGCC	$(ATAG)_5$	58
RM140*	TGCCTCTTCCCTGGCTCCCCTG GGCATGCCGAATGAAATGCATG	$(CT)_{12}$	58

*polymorphic between parents

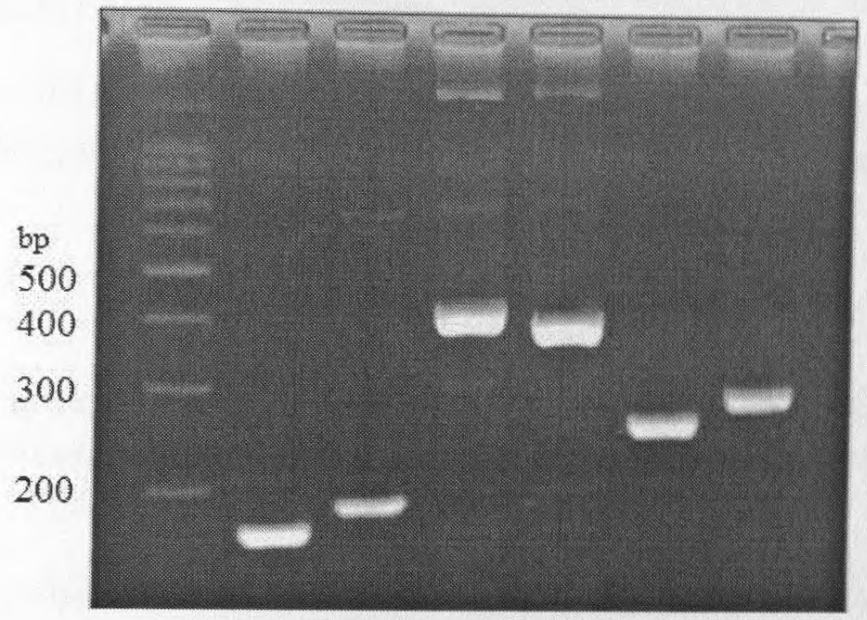

Figure 01: Polymorphism between parents, At354 and Bg352

Lane L- 100 bp DNA ladder, Lane 1,3 and 5- At354 amplified with RM10852, RM10772 and RM140 respectively, Lane 2,4 and 6- Bg352 amplified with RM10852, RM10772 and RM140 respectively

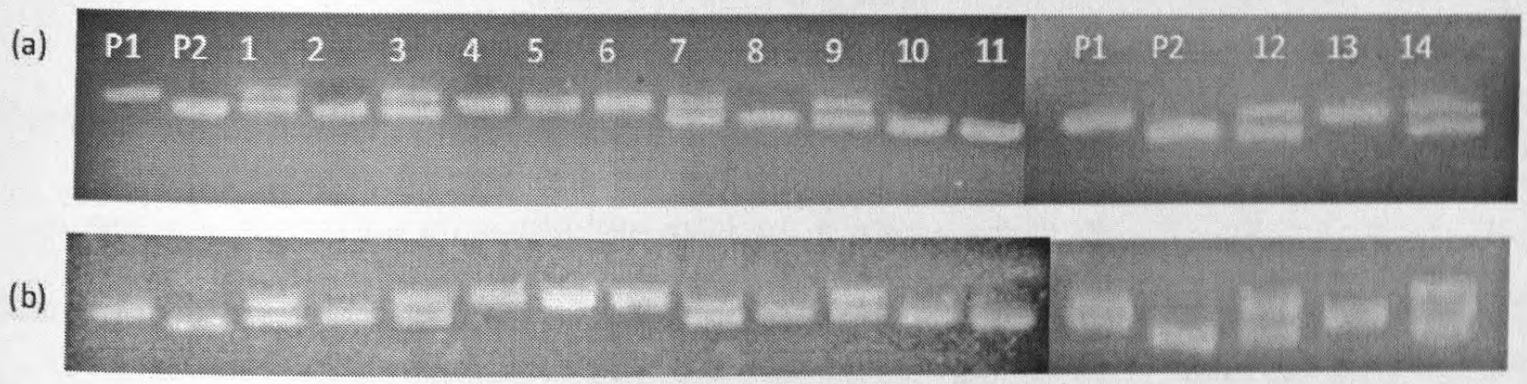

Figure 02: PCR profile generated with F_1 individuals from (a) RM1287, (b) RM493 SSR markers.

Lane P1- At354 parent, Lane P2-Bg352 parent, Lane 1-14- F_1 individuals

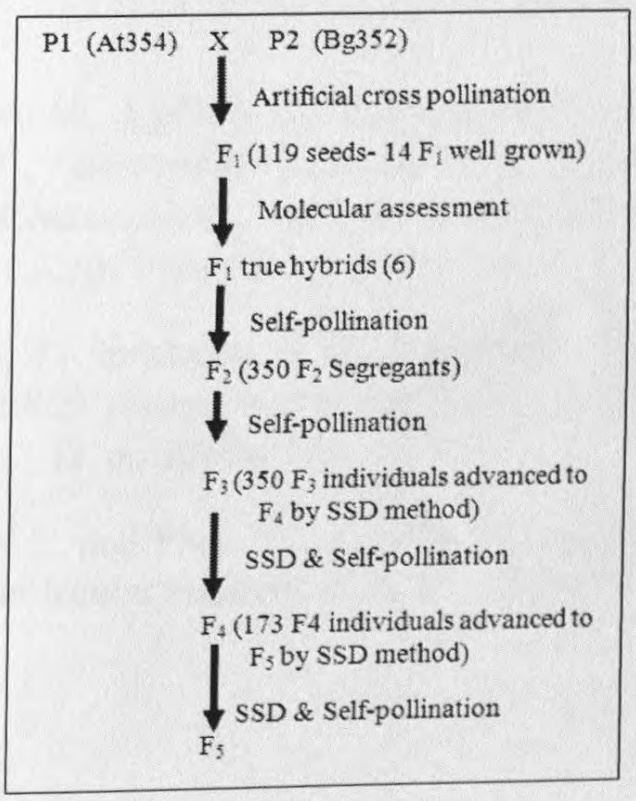

Figure 03: Schematic representation of the progress of developing mapping population

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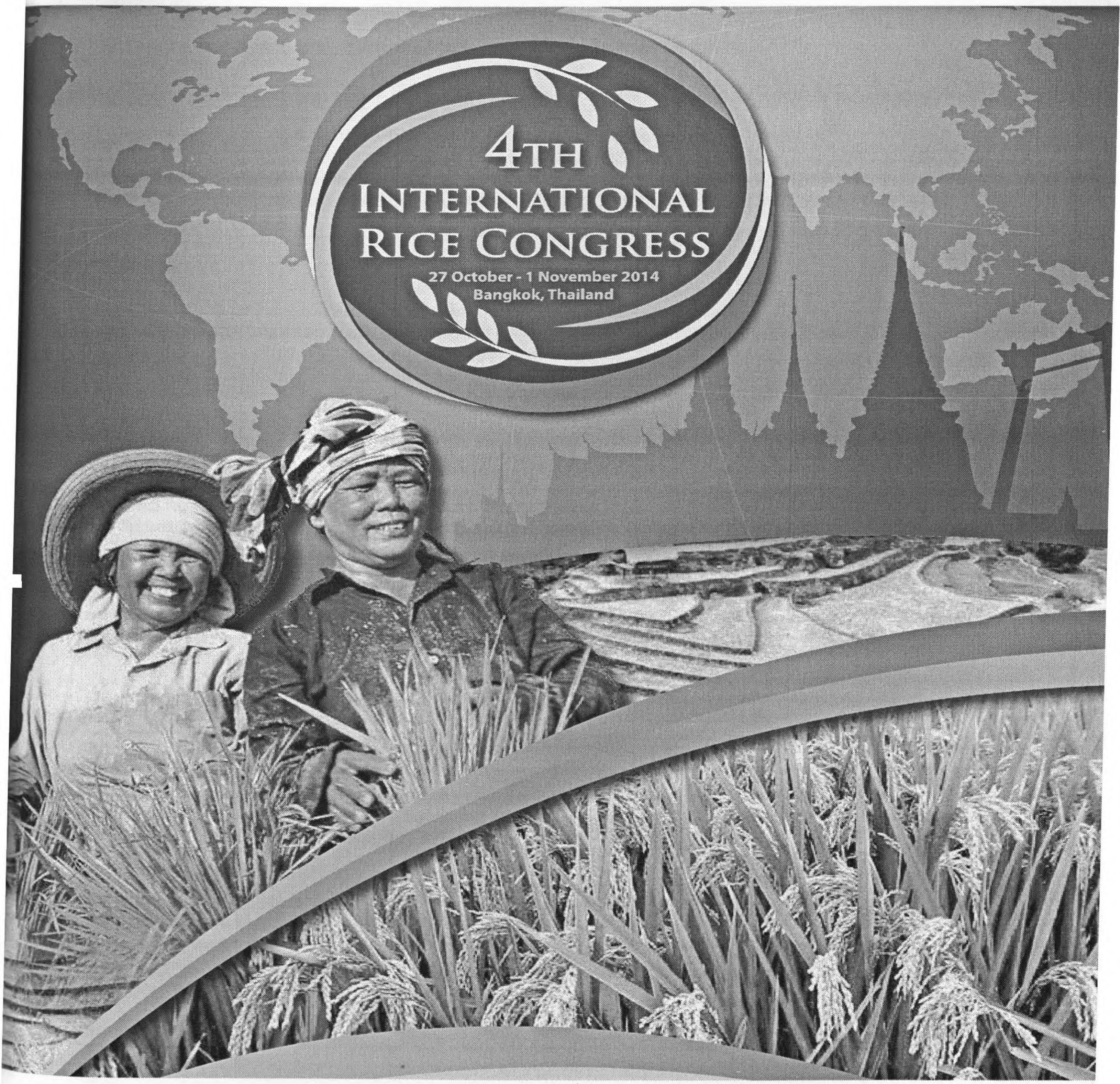
Annexure 07

4TH INTERNATIONAL RICE CONGRESS

27 October - 1 November 2014
Bangkok, Thailand



IRC14-0834
02b. Improved donors and genes/QTL conferring valuable traits

MAPPING OF SALINITY TOLERANT QTLS ON CHROMOSOME 1 IN RICE (ORYZA SATIVA) USING RILS DERIVED FROM AT354 AND BG352

R. Dikkumburage[1], *N.S. Kottearchchi*[1], *W.L.G. Samarasinghe*[2], *G.B. Gregorio*[3]
[1]*Department of Biotechnology, Wayamba University of Sri Lanka, Makandura, Sri Lanka*
[2]*Biotechnology, Rice Research and Development Institute, Batalagoda, Sri Lanka*
[3]*Plant Breeding Genetics and Biotechnology Division, International Rice Research Institute, Los Banos, Sri Lanka*

Purpose:
Salinity is one of the main soil problems impeding rice production in Sri Lanka. Breeding for salt tolerance is the most promising approach to enhance the productivity in saline prone areas. Salinity tolerance is a quantitative trait, governed by many genes across the rice genome making complexity in breeding. Present study was initiated to identify salinity tolerant QTLs in elite rice background, facilitating the breeding for salt tolerance.

Approach and methods used:
At354 (Pokkali/Bg94-1) and Bg352 were selected as salt tolerant and susceptible parents respectively. Recombinant inbred lines (RILs) population (300 lines) was developed as a mapping population through single seed descent method. Of them 100 RILs were screened phenotypically for seedling stage salinity tolerance using hydroponics system under EC of 12dSm^{-1}. Population was assessed using Salinity Survival Index (SSI), length and dry weight of shoots and roots.

Previously, a major QTL (*Saltol*), was identified on chromosome 1 using RILs derived from IR29/Pokkali cross. Therefore, study initially focused on *Saltol* region as At354 has the background of Pokkali. Accordingly, parents were screened using 42 SSR markers associated with the *Saltol* region and RILs were genotyped using polymorphic markers. Subsequently, linkage map was constructed and QTL analysis (simple interval mapping) was performed.

Key results:
Out of 42 markers surveyed, 12 markers were polymorphic between parents. RILs were genotyped using polymorphic markers and linkage map was constructed covering the length of 7.3cM at an average 2 locus interval of 1.46cM. One significant QTL, qRSS1.1, was detected for SSI flanking RM10852 and RM10864 markers at about 14 Mb on the short arm of the chromosome 1 closer to *SalT* gene, explaining 6.2% of phenotypic variation.

Synthesis and Applications:
QTL identified in this study would be useful to be introgressed into elite cultivars facilitating the breeding for salt tolerance. Further studies are being continued to saturate the genetic map using more number of SSR and SNP markers to detect closer association to the salinity tolerance with the reported QTLs and other novel QTLs.

Annexure 08

IRC14-0565
02b. Improved donors and genes/QTL conferring valuable traits

ANALYSIS OF QTL FOR SALINITY TOLERANCE ON CHROMOSOME 4 IN RICE

B.A. Dahanayaka[1], *N.S. Kottearachchi*[1], *D.R. Gimhani*[1], *W.L.G. Samarasighe*[2]
[1]*Department of Biotechnology, Wayamba University of Sri Lanka, Makandura Gonawila, Sri Lanka*
[2]*Department of Plant Breeding and Biotechnology, Rice Research and Development Institute, Bathalegoda Ibbagamuwa, Sri Lanka*

Purpose:
Development of salt resistant rice varieties has been a current requirement as many rice growing countries suffer from saline affected soil. Recognizing of salt tolerant QTLs from different varieties and compiling them into a one line would be a promising way to develop salt resistant varieties. Aiming at achieving this objective, we used a Recombinant Inbred Line (RIL) population previously developed from the cross between At354, a salt tolerant variety and Bg 352, a salt susceptible variety to explore the QTLs for salinity tolerance spanning few chromosomes. Here we report a novel QTL and SSR markers associated with the seedling tolerance under salt stress.

Approach and methods used:
Phenotyping was performed in hydroponics with salt stress of 12 dS/m using 100 lines of F5 population. At seedling stage, Salinity Survival Index (SSI), root length, shoot length, dry shoot weight and dry root weight were assessed under salt stress. Parents were surveyed for the polymorphism using SSR markers in few chromosomes and the SSR markers that were polymorphic were used for genotyping of the F5 lines.

Key results:
Statistical analysis enabled us to detect few SSR makers that are significantly linked with phenotypic traits. Among them, chromosome 4 showed a putative novel QTL (qRSS4.1) that significantly affect for SSI ($P< 0.01$), shoot length ($P<0.01$), Root length ($P<0.05$), and Dry root weight ($P<0.05$). QTL map was constructed and the putative QTL was significant explaining 9% phenotypic variation in SSI.

Synthesis and Applications:
This QTL is worth to be exploited for fine mapping and gene discovery in order to utilize in marker assisted breeding in rice.

Acknowledgment: Financial assistance from National Science Foundation of Sri Lanka (RG/2011/BT/02)

Annexure 09

IRC14-0975
01e. Characterization and evaluation of rice genetic diversity, including 'omics'

ASSESSMENT OF SEEDLING TOLERANCE OF SRI-LANKAN RICE (ORYZA SATIVA) GERMPLASM UNDER SALT STRESS USING SSR MARKERS LINKED WITH SALTOL QTL

N.S. Kottearachchi[1], *B.A. Dahanayaka*[1], *D.R. Gimhani*[1]
[1]*Department of Biotechnology, Wayamba University of Sri Lanka, Makandura, Sri Lanka*

Purpose:
There are vast numbers of divergent rice germplasms in Sri Lanka. As the development of salinity tolerant cultivars has become a prioritized research area, it is necessary to assess these varieties and utilize them in breeding programs. Previously a major salinity tolerant QTL called *Saltol* has been discovered in chromosome 1 of Pokkali derived germplams. Assessment of SSR markers at the region of previously reported QTL, *Saltol,* and phenotypic assessment of seedling stage of rice germplasms would give some insight into the understanding of salt tolerance

Approach and methods used:
In this regard we analyzed twenty rice germplasms, including traditional and improved varieties, with five SSR markers closely linked to *Saltol* QTL. We derived a novel formula of Salinity Survival Index (SSI) in order to assess the survivability of the plants under stress in terms of quantitative parameter. Morphological traits of the seedlings were assessed under saline condition which was created with 100mM NaCl concentration (12 ds/m) in hydroponics.

Key results:
The variation of survivals among germplasm was easily assessed as the SSI distributed quantitatively. Results showed that SSI, root length, shoot length, fresh root weight, and dry root weight, were significantly different among varieties. SSI and visual injuries were exhibited that some traditional varieties and exotic varieties are extremely tolerant even than Pokkali, the well-known salt tolerant check variety. The dendrogram obtained from cluster analysis of DNA markers indicated that some of the tolerant varieties were grouped in separate clusters.

Synthesis and Applications:
Polymorphic banding patterns of SSR markers obtained from tolerant and susceptible varieties near *Saltol* would be useful in selecting parental lines for the rice improvement breeding programs designed for salt tolerance.

Acknowledgment: Financial assistance from National Science Foundation (RG/2011/BT/02)

Annexure 10

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1st Rajhuna International Science & Technology Conference
University of Rajhuna, Matara, Sri Lanka
January 22-23, 2014

RJSTCON 2014

Allelic diversity and seedling tolerance of some rice (*Oryza sativa*) germplasms under salt stress

B.A. Dahanayaka[1], N.S. Kottearachchi[1]*, D.R. Gimhani[1] and W.L.G. Samarasighe[2]

[1]*Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, Makandura, Gonawila (NWP), Sri Lanka*
[2]*Rice Research and Development Institute, Bathalegoda, Ibbagamuwa, Sri Lanka*

There are vast numbers of divergent rice germplasms in Sri Lanka. As the development of salinity tolerant cultivars has become a prioritized research area, it is necessary to assess these varieties and utilize them in breeding programs. Previously a major salinity tolerant QTL called *Saltol* 1 has been discovered in chromosome 1 of Pokkali derived germplams. Assessment of SSR markers at the region of *Saltol 1* QTL and phenotypic assessment of seedling stage of rice germplasms would give some insight into the understanding of salt tolerance. In this regard, we analyzed twenty rice germplasms, including traditional and improved varieties, with five SSR markers closely linked to *Saltol* 1. Also morphological traits of the seedlings were assessed under saline condition which was created with 100mM NaCl concentration (12 ds/m) in hydroponics. Results showed that root length, shoot length, fresh root weight, and dry root weight were significantly different among varieties. Survival index and visual injuries were exhibited that some traditional varieties and exotic varieties are extremely tolerant even than Pokkali, the well-known salt tolerant check variety. The dendrogram obtained from cluster analysis of DNA markers indicated that some of the tolerant varieties were grouped in separate clusters. Polymorphic banding patterns of SSR markers obtained from tolerant and susceptible varieties near *Saltol 1* would be useful in selecting parental lines for the rice improvement breeding programs designed for salt tolerance.

Key words: Rice germplasms, salinity tolerance, *Saltol 1* QTL, SSR markers

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*kottearachchins@yahoo.com

Annexure II

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PROGRESS TOWARDS MAPPING OF A QTL FOR SALINITY TOLERANCE ON CHROMOSOME 4 IN RICE

B.A. Dahanayaka[1], N.S. Kottearachchi[1], D.R. Gimhani[1], and W.L.G. Samarasighe[2]

[1]Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, Makandura, Gonawila (NWP); [2]Rice Research and Development Institute, Bathalegoda, Ibbagamuwa

Corresponding author: kottearachchins@yahoo.com

In Sri Lanka, rice cultivation in coastal areas has been affected by the saline ground water especially in the dry season. Cultivation of salt resistant rice varieties is the most feasible and promising way to survive under saline soil. Salt tolerance is a polygenic character, governed by Quantitative Trait Loci (QTLs) scattered throughout the whole genome of rice. Hence, it is necessary to identify salt tolerant varieties that have accumulated different salt tolerant QTLs using molecular breeding programs. Aiming at achieving this objective, a Recombinant Inbred Line (RIL) population was previously developed by crossing of At354, a salt tolerant variety with Bg352, a salt susceptible variety. At354 is an elite rice variety originated from Pokkali which has been frequently used as a donor of salt tolerant genes in breeding programmes. Therefore, it can be assumed that Pokkali inherited QTLs might be present in salt resistant RIL lines. In this study we assessed 88 SSR markers that are located about 30-40 cM apart from each other in several chromosomes for the polymorphisms between two parents. Seedlings of 100 F5 lines were grown under 12EC level of the salinity in hydroponics and Seedling Survival Index was determined in each line. Of all SSR markers tested 19 markers were found to be polymorphic between parents. In Chromosome 4 seven polymorphic SSR markers were detected and a linkage map was constructed. One SSR marker located in chromosome 4 showed that At354 genotype has significantly high salinity survival index (0.01) contributing to the salt tolerant phenotype. Hence a significant QTL for salinity tolerance was found in chromosome 4 of At354 and Bg352 cross. This is the first reported QTL identified in Sri Lanka using locally bred mapping population of rice.

The financial assistance from National Science Foundation (RG/2011/BT/02) is gratefully acknowledged.

Keywords: Rice, Salinity Survival Index, SSR markers, QTL

P0211

INVESTIGATION OF TRIAZOLE PESTICIDES PROPICONAZOLE, TEBUCONAZOLE AND FENBUCONAZOLE FOR THEIR ABILITY TO SUPRESS THE DEVELOPMENT OF BAKANAE DISEASE ON RICE IN IN VITRO CONDITIONS

I. Karov[1], S. Mitrev[1], B. Kovacevik[1], E. Kostadinovska[1]

[1]*Faculty of Agriculture Department for plant and environment protection, University of Goce Delcev - Stip, Stip, Macedonia*

The efficiency of triazine pesticides propiconazole, fenbuconazole and tebuconazole against the bakanae disease on rice was evaluated in this study in in vitro conditions. The first screening was made at concentrations of 1 mg/l, 5 mg/l, 10 mg/l and 15 mg/l. Then, concentrations are differentiated in order to find the best effective dose for the suppression of the disease. The some dose of each investigated pesticide was also investigated for the possibility of phytotoxic effect on the early stages of plant development. The obtained results shows that all three investigated pesticides are effective,not toxic for the plant when applied in the effective doses and can be acceptable for the suppression of bakanae disease on rice in the early stages of development.

P0212

DEVELOPMENT OF MAPPING POPULATION FOR SALINITY TOLERANCE USING AT354 AND BG352 RICE VARIETIES

R. Dikkumburage[1], N.S. Kottearachchi[1], W.L.G. Samarasinghe[2]

[1]*Department of Biotechnology, Wayamba University of Sri Lanka, Makandura, Sri Lanka*
[2]*Department of Biotechnology, Rice Research and Development Institute, Batalagoda, Sri Lanka*

Introduction
Development of Salinity tolerance in rice is one of the main breeding requirements at present in Sri Lanka. Salinity tolerance in rice is a quantitative trait which is governed by many genes across the rice genome. Development of mapping population for salt tolerance is a prerequisite for the identification of salinity tolerant QTLs. Therefore, an attempt was made to develop a mapping population using two elite rice varieties that exhibit two extremes.

Methodology
At354, salt tolerant parent and Bg352, salt susceptible parent were crossed as an attempt of developing mapping population for salt tolerance. Hybridity of F_1 progeny was assessed using SSR markers. Subsequent generations were developed by single seed decent method and homogeneity of the population was assessed.

Results
SSR markers RM1287, RM493, RM8094, RM10764, RM10772, RM10745, RM3412 RM140, RM10852, RM10864, RM10694 and RM10793 exhibited polymorphism between At354 and Bg352. Hybridity was assessed using parental polymorphic markers and 6 true hybrids were selected. By selfing of true hybrids, 350 F_2 segregants were raised successfully followed by advancing of subsequent generations. Homogeneity of the population was confirmed by polymorphic SSR markers using F_5 generation.

Discussion and Conclusion
This study explained the feasibility of utilizing microsatellite markers for verification of hybridity and population development in At354 and Bg352 or related crosses in rice breeding programmes. Polymorphic SSR markers would be useful in construction of Linkage and QTL maps.

7th INTERNATIONAL RICE GENETICS SYMPOSIUM
5 - 8 November 2013 • Dusit Thani Hotel, Manila, Philippines

P0213

ZHONGZU14:A POTENTIAL GREEN SUPER RICE GENOTYPE FOR WATER SAVING CONDITION UNDER IRRIGATED ECOSYSTEM

H. Ahmed[1], S. Akhtar[1], H. Khatun[1], T. Ansari[1], J. Ali[1]

[1]*Plant Breeding Division, Bangladesh Rice Research Institute, Dhaka, Bangladesh*

Water will be a limiting factor in coming days and becoming expensive and increase rice production cost. Genetically potential water saving genotype is needed to reduce rice production cost. Several advanced lines were evaluated in farmersÕ field followed by Participatory Varietal Selection (PVS) process since 2009-10 dry season in Bangladesh. In 2010-11 dry season, five genotypes were selected by farmers in PVS field among them Zhongzu14 got higher farmers votes. In 2011-12 dry season, trials were conducted at different locations both under irrigated and control drought (irrigation was limited after four weeks of transplanting). Under irrigated condition at Gazipur, Zhongzu14 yielded lower than the check variety BRRI dhan28 where under drought condition in the North-Western region Zhongzu14 yielded higher than the check. The laboratory screening also reveals that yield of Zhongzu14 decrease by 19.7% under 70% soil saturation while 13.7% increased under saturated condition compared to irrigated control condition where the popular check variety BRRI dhan28 found 8.23% less yield under saturated and 64.4% less yield under 70% saturation indicating Zhongzu14 is a genotype can grow under less water and save water for future generation. This line will be high yielding potential water saving rice variety in future.

Note: 1: Bangladesh Rice Research Institute, 2: International Rice Research Institute

P0214

PHYSIOLOGICAL RESPONSES TO HEAT STRESS IN (ORYZA SATIVA L.):,SCREENING OF NITROGEN, CHLOROPHYLL CONTENT AND CELL MEMBRANE STABILITY OF FLAG LEAF

M. Mombeini[1], R. Abdol Ali Gilani[1]

Annexure 13

Microsatellite Marker-based Hybridity Assessment; an approach towards development of mapping population for salinity tolerance in rice

D. R. Gimhani[1*], N. S. Kottearachchi[1] and W. L. G. Samarasinghe[2]

1 Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba Univesity of Sri Lanka
2 Rice Research and Development Institute, Batalagoda, Sri Lanka.

Salinity tolerance in rice is a quantitative trait which is governed by many genes. Present study was initiated with the aim of identifying salinity tolerant quantitative trait loci (QTLs) in elite rice background under Sri Lankan environment. In view of this, development of mapping population for salt tolerance is a prerequisite for the identification of salinity tolerant QTLs.

In rice, getting of true hybrids is a technical problem as self- fertilization may occur before out crossing. Conventional selection of hybrids based on specific morphological traits is time consuming, restricted to a few characteristics, influenced by environmental condition and inefficient. Therefore, present study was conducted for the precise assessment of hybridity in F1 progeny developed for salt tolerance as the assessment of hybridity is a crucial factor for subsequent advancing of breeding progenies.

At354 variety derived from Pokkali and Bg94-1 was selected as salt tolerant parent as it exhibits resistant to salinity in Sri Lanka while Bg352 was selected as a salinity susceptible cultivar. By crossing At354 with Bg352, F_1 progeny consisting of 119 F1 seeds was developed. Parents were genetically screened for polymorphism using 16 microsatellite markers associated with the region of *Saltol* QTL in chromosome 1 and out of those, 9 markers RM1287, RM493, RM8094, RM10764, RM10745, RM10772, RM3412, RM10852 and RM140 exhibited parental polymorphism. Of them, RM 1287 and RM 493 which were closer to the *Saltol* QTL were used to examine 14 well grown F1 individuals and their hybridity was confirmed by comparing complementary alleles of parents. Accordingly, hybridity of 6 F_1 individuals was confirmed proving their heterozygosity at the respective loci. By selfing of 6 true hybrids, 350 F_2 segregants were raised successfully followed by advancing of subsequent generations towards development of recombinant inbred lines which could be utilized as mapping population for identification of salinity tolerant QTLs under Sri Lankan environment. This study proved the feasibility of utilizing RM 1287 and RM493 markers for verification of hybridity in future breeding programmes which utilize same parental lines.

Keywords: Hybridity assessment, microsatellite markers, rice, salinity tolerance

Acknowledgements: *Financial Assistance from Wayamba University of Sri Lanka (SRHDC/RP/01/10-05) and NSF Grant (RG/2011/BT/02)*

Annexure 14

Nugaliyadda L., Marambe B., Ratnasekera D., Silva P., Nanayakkara C., Rajapakse R. and Deshmukh A. (editors) (2012) Book of Abstracts, Sri Lanka India Conference on Agro Biotechnology for Sustainable Development, 12-13 March, B M I C H, Colombo, Sri Lanka

Mapping population development for salinity tolerant QTLs in rice and microsatellite marker-based hybridity assessment

Gimhani D.R.[1], Kottearachchi N.S. [1]and Samarasinghe W.L.G.[2]

[1] Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba Univesity of Sri Lanka, Makandura, Gonawila (NWP), Sri Lanka.

[2] Rice Research and Development Institute, Batalagoda, Ibbagamuwa, Sri Lanka.

Salinity tolerance in rice is a quantitative trait, which is governed by many genes. Therefore, the present study was initiated with the aim of identifying salinity tolerant quantitative trait loci (QTLs) in elite rice background under Sri Lankan environment in order to develop salinity tolerant cultivars. Rice (*Oryza sativa*) variety At354, derived from Pokkali and Bg94-1 cross, exhibits extreme salinity tolerance and thus, was selected as the salinity-tolerant parent while Bg352 was selected as a susceptible parent. The At354 was crossed with Bg352 and 119 F_1 seeds were produced. In rice, getting of true hybrids is a technical problem as self-fertilization may occur before out crossing, preventing the transfer of the desired traits in progenies. In addition, the conventional selection of hybrids based on specific morphological traits is time consuming, restricted to a few characteristics, influenced by environmental condition and inefficient. Therefore, a molecular approach was followed for precise assessment of hybridity in F_1 generation by using microsatellite markers associated with the major salt tolerant QTL (*Saltol*). The selected parents were genetically screened along with the variety Pokkali using RM1287, RM562, RM493, RM6711, RM8115, RM10745 and RM10772 microsatellite markers. Out of those, RM10745, RM10772 and RM1287 were polymorphic between parents and co-segregate with 'Pokkali indicating inheritance of Pokkali origin chromosome 1 region in At354. Using polymorphic RM1287, 14 F_1 individuals that were grown successfully were examined and out of them, hybridity of 6 F_1 individuals was confirmed. By selfing of six true F_1 hybrids, 350 F_2 segregants were raised successfully followed by advancing of subsequent generations by single seed descent method in order to produce recombinant inbred lines (RILs). Future studies are necessary to be utilized At354 x Bg352 RILs as mapping population for identification of salinity tolerant QTLs under Sri Lankan environment.

Keywords: Salinity tolerant rice, microsatellite markers, hybridity assessment

Please register **before 14th August** by sending the filled registration form to
madhavid@wyb.ac.lk

Registration form can be downloaded from Wayamba University of Sri Lanka website,
http://www.wyb.ac.lk

Confirmation of the Participation:

Confirmation will be on first come first serve basis...

After registration, we will notify the successful registrants to confirm their participation by paying the registration fee to Wayamba University of Sri Lanka (Wayamba University of Sri Lanka, A/C No. 137100120000963, People's Bank, Makandura). Please send the bank slip along with the hard copy of the filled registration form to the;

Head
Department of Biotechnology
Faculty of Agriculture and Plantation Management,
Wayamba University of Sri Lanka,
Makandura, Gonawila (NWP).

And

Mention "Workshop on Gene/QTL mapping in Plants" on top left side corner of the envelop

- Please consider that confirmation of the registration will be done only after receiving the payment.

For more Details, Contact:

Ms. Madhavi P. Dassanayaka,
Department of Biotechnology
Tel No. : 076 - 7576376
E-mail : madhavid@wyb.ac.lk

WORKSHOP ON GENE/QTL MAPPING IN PLANTS

Organized by

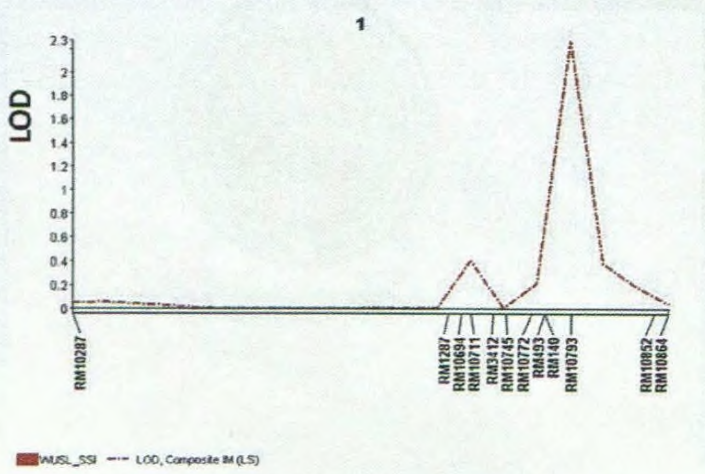
Department of Biotechnology
Faculty of Agriculture and Plantation Management
Wayamba University of Sri Lanka

08th and 09th September 2015

Annexure 15

Although Sri Lanka is endowed with a divergent crop germplasm, these resources have not been utilized much in modern breeding programs mainly because their genetic status is unrevealed to the breeders. QTL mapping based approach is one of the promising ways in finding out genes for many traits such as yield, quality and abiotic and biotic stress tolerance. Therefore, this workshop has been planned with the long-term objective of utilizing favorable genes of divergent germplasm in Sri Lanka for breeding of improved varieties.

In this regard we invite students, researchers, breeders or any beginner of this subject to gain the knowledge on QTL/Gene mapping in simplified manner, so that they can make use of this knowledge directly in breeding purposes for crop improvement, proposal preparation to obtain research grants and for initiation of collaborative research programs etc.



- Application of molecular markers in MAS and gene/ QTL/mapping
- Plant populations for gene/QTL mapping
- Marker- trait association analyses for quantitative traits (Principle of QTL mapping)
- Hands-on experience with linkage mapping software
- Hands-on experience with QTL mapping software
- Hands -on experience with mapping genes/QTLs by single marker analysis, Simple interval mapping and Composite interval mapping
- Gene/QTL mapping in cereals: A case study on mapping for salt tolerant genes in rice
- Gene mapping techniques for tree crops: A case study with Coconut
- Substitution mapping : A case study with *Arabidoposis thaliana*
- Overview of automated genotyping with SNP markers

Participants are requested to bring along laptop computers, if you need to load the gene mapping programs (Qgene 4.3.10, WinQTLCart and JoinMap4.1) to your computers!!

8th and 9th September 2015

Venue:

Staff Development Centre,
Faculty of Agriculture and Plantation Management,
Wayamba University of Sri Lanka
Makandura, Gonawila

Registration Fee: Rs. 5000/=

(Include lunch, refreshments, presentation handouts and stationery)

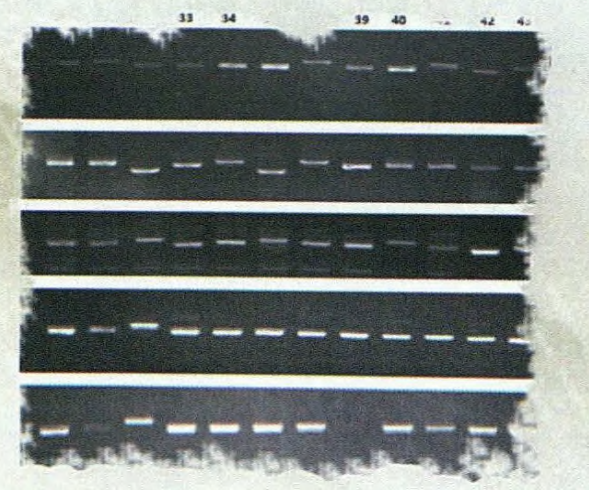
Accommodation:

Participants are advised to arrange the accommodations by themselves in advance.
Possible places for accommodation:

University Guest House, Makandura
Tel : 031 - 2298111

Guest House in Regional Agricultural Research & Development Centre, Makandura,
Tel: 031 - 2299625, 031 - 12299805

Hostels in Coconut Development Training Center, Lunuwila.
Tel : 031 - 2255048, 076 - 6905125



Annexure 16

Workshop on Gene /QTL mapping in plants

8th -9th September 2015

Venue: Staff Development Centre, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka (WUSL)

Day 1: 8th September 2015		
		Resource Personnel
8.30 -900	Registration + Refreshments	
9.00 -9.05	Welcoming address by Head of the Department of Biotechnology	
9.05 - 9.15	Address by Vice chancellor of WUSL	
9.15 -9. 30	Self introduction by participants and resource persons	
9.30 -10.00	Application of Molecular markers (MM) in Marker Assisted Selection (MAS) and QTL/gene mapping *Desired outcomes:* Overview of molecular markers , Examples for the use of MM in MAS, Importance of MM in QTL/gene mapping	Prof. Thilak Attanayaka, WUSL
10.00 - 10.30	Introduction to genome mapping and mapping populations *Desired Outcomes:*Brainstorming on requirements for genome mapping, types of mapping populations and applicability of each mapping population	Dr. Chandrika Perera, CRI
Working snack		
10.30 -11.15	Principle of QTL/gene mapping *Desired Outcomes:* Introducing the necessity for genotyping and phenotyping of mapping populations, Marker trait association analysis for quantitative traits, Brainstorming of Linkage mapping and QTL/gene mapping methods	Ms DR Gimhani,WUSL
11.15-1,15	Practical: Hands on experience with linkage mapping *Desired Outcomes:*Introducing scoring of genotypes, overview of linkage mapping	Ms DR Gimhani, Ms BuddhikaDahanayake (WUSL), Prof. N. Kottearachchi,

	software and construction of linkage maps by their own using Join map software	Dr.Chandrika Perera
1.15 2.15 Lunch break		
2.15 -4.00	Practical Continuation -Hands on experience with linkage mapping	Ms DR Gimhani, Ms BuddhikaDahanayake, Prof. N. Kottearachchi, Dr.Chandrika Perera
Working snack		
4.00 -4.30	A Case study on mapping for salt tolerant genes in rice *Desired Outcomes:* Introducing a plan for a QTL mapping study based on a cereal crop and implementation of a QTL mapping project	Prof. N. Kottearachchi, WUSL

Day 2: 9th September 2015		
9.00 - 10.00	Gene mapping techniques for tree crops *Desired Outcomes:*A case study with coconut and genome wide association study (GWAS)	Dr. Chandrika Perera, CRI
Working snack		
10.00 - 12.30	Practical: Hands on experience with mapping genes/QTL by single marker analysis, Simple interval mapping (SIM) and composite interval mapping (CIM) *Desired Outcomes:* Overview of QTL mapping software i.e Qgene and Win QTL Cartographer, Operating Qgene software with the provided datasheets sothat participant can construct QTL maps by their own	Ms DR Gimhani, Ms BuddhikaDahanayake, Prof. N. Kottearachchi, Dr.Chandrika Perera
12.30 - 1.30 Lunch break		
1.30 - 2.30	Practical continuation: Mapping genes/QTLs Composite Interval mapping	Ms DR Gimhani, Ms BuddhikaDahanayake, Prof. N. Kottearachchi, Dr.Chandrika Perera
2.30- 3.15	Approaches for fine mapping, NILS, CSL *Desired Outcomes:*Construction of special populations and use of them for more precise/fine mapping of QTLs/genes	Dr. Chandrika Perera, CRI
Working snack		

3.15-3.40	Overview of automated genotyping with SNP markers *Desired Outcomes:* Introduction to high throughput SNP genotyping platforms, underlying principle of the technique in brief and usefulness of the technique in molecular breeding	Ms DR Gimhani, WUSL
3.40 -4.00	Implementation of the outcomes of QTL mapping *Desired Outcomes:*Brainstorming on final outcomes of QTL maps leading to candidate gene discovery and developing introgressed lines using examples	Prof. N.Kottearachchi, WUSL
4.00- 4.30	Participants feedback and Awarding of certificates	Prof. Thilak Attanayaka, WUSL
End of the workshop		

Contact details of Participants - Workshop on Gene/QTL Mapping

On 8th and 9th September 2015

Organized by

Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka

No.	Name of the registrant	Institute	Designation
01.	Ms. K. N. S. Perera	Coconut Research Institute, Lunuwila	Ph.D. student
02.	Ms. Liyanage Chandima Jayamali Kamaral	Genetech molecular Diagnostics (Genetech)	Scientist
03.	Ms. WWMA Iroshini	Coconut Research Institute, Lunuwila.	MPhil student
04.	Mr. Y.A. Prasanna Kumara Dayasena	Postgraduate Institute of Science, University of Peradeniya.	Postgraduate student
05.	Dr. (Mrs.) Samanthi Priyanka Witanage	Department of Genetics and Plant Breeding Rubber Research Institute.	Principle Research Officer, Head/ Department of Genetics and Plant Breeding
06.	Ms. PV Asiri Anushka	Department of Genetics and Plant Breeding Rubber Research Institute	Research officer
07.	Ms. Charitha Janaki Gunasekara	Faculty of Science,	Undergraduate

Annexure 17

		University of Colombo	
08.	Mr. H.A.S.D. Perera	University of Ruhuna	Research Assistant
09.	Ms. Elpitiya Udari Uvindhya Rathnathunga	University of Ruhuna	PhD student / Research assistant
10.	Mr. S.W.G.C. Ruwan Kumara	Coconut Research Institute, Lunuwila	Research Officer
11.	Ms. Anasuya NishamaniKodithuwakku	Department of Agricultural Biology, Faculty of Agriculture, University of Ruhuna.	Research Assistant/ Post Graduate Student
12.	Ms. Nadugala Vithanage Thilini Jayaprada	Department of Agricultural Biology, Faculty of Agriculture, University of Ruhuna.	Research Assistant/ Post Graduate Student
13.	Mr. Nandakumar Jeyadarshan	Coconut Research Institute, Lunuwila	Research officer
14.	Dr. H D D Bandupriya	Coconut Research Institute, Lunuwila	Senior Research officer
15.	Ms. Gampolawaduge Gayani Manjula Fonseka	Institute of Biochemistry Molecular Biology and Biotechnology (IBMBB)	MSc. Student
16.	Ms. Alawaturage Malika Marian Sudarshini Perera	Sugarcane Research Institute, Uda Walawe.	Research Officer
17.	Dr. Sudarshanee Geekiyanage	University of Ruhuna	Senior Lecturer
18.	Ms. Duvini Chathurangi Padukkage	Faculty of Graduate Studies, Faculty of Agriculture, Board of Study in Agriculture,	Research Assistant/M.Phil. candidate

		University of Ruhuna	
19.	Ms. Rumana Azad	Department of Agricultural Biology, Faculty of Agriculture, University of Ruhuna,	PhD student
20	Ms. Tolshi Taniya Dias Dahanayake	Faculty of Agriculture , University of Ruhuna	Temporary Demonstrator
21	Ms. Sumithra Arachchige Don. Pradeepa Sandamali Jayawardhana	Biochemistry Division, Tea Research Inatitute	Research Officer
22.	Dr. Aruna Wijesuriya	Sugarcane Research Institute	Principal Research Officer and Head of Crop Improvement Division

Workshop on Gene/QTL Mapping
On 8th and 9th September 2015

Registration Sheet: Day 01 (08.09.2015)

No.	Name of the registrant	Institute	Signature
01.	Ms. K. N. S. Perera	Coconut Research Institute	
02.	Ms. Liyanage Chandima Jayamali Kamaral	Genetech molecular Diagnostics	
03.	Ms. W.W.M.A. Iroshini	Coconut Research Institute	
04.	Mr. Y.A. Prasanna Kumara Dayasena	Postgraduate Institute of Science, University of Peradeniya	
05.	Dr. (Mrs.) Samanthi Priyanka Witanage	Rubber Research Institute	
06.	Ms. P.V. Asiri Anushka	Rubber Research Institute	
07.	Charitha Janaki Gunasekara	Faculty of Science, University of Colombo	
08.	Mr. H.A.S.D. Perera	University of Ruhuna	
09.	Ms. Elpitiya Udari Uvindhya Rathnathunga	University of Ruhuna	
10.	Mr. S.W.G.C. Ruwan Kumara	Coconut Research Institute	
11.	Ms. Anasuya Nishamani Kodithuwakku	University of Ruhuna	
12.	Ms. Nadugala Vithanage Thilini Jayaprada	University of Ruhuna	
13.	Mr. Nandakumar Jeyadarshan	Coconut Research Institute	

14.	Dr. H.D.D. Bandupriya	Coconut Research Institute	
15.	Ms. Gampolawaduge Gayani Manjula Fonseka	Institute of Biochemistry Molecular Biology and Biotechnology	
16.	Ms. Alawaturage Malika Marian Sudarshini Perera	Sugarcane Research Institute	
17.	Dr. Sudarshanee Geekiyanage	University of Ruhuna	
18.	Ms. Duvini Chathurangi Padukkage	University of Ruhuna	
19.	Ms. Rumana Azad	University of Ruhuna	
20	Ms. Tolshi Taniya Dias Dahanayake	University of Ruhuna	on the table.
21	Ms. Sumithra Arachchige Don. Pradeepa Sandamali Jayawardhana	Tea Research Institute	
22.	Dr. Aruna Wijesuriya	Sugarcane Research Institute	

Annexure 19

IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE

OFFICE OF THE DEPUTY DIRECTOR GENERAL (RESEARCH)

2011 October 17

MS. DIKKUMBURAGE RADHIKA GIMHANI
Lecturer
Department of Biotechnology
Faculty of Agriculture and Plantation Management
Wayamba University of Sri Lanka
Makandura, Gonawila, 60170, Sri Lanka
E-mail Address:

Dear Ms. Gimhani:

I am pleased to inform you that you have been awarded the **2011 Global Rice Science Scholarship** to enable you to pursue your PhD Sandwich Degree Program at the Wayamba University of Sri Lanka and IRRI under the supervision of **Dr. Glenn Gregorio**, Senior Scientist, Plant Breeding, Genetics and Biotechnology (PBGB) Division. Under the terms of this invitation you are expected to be enrolled at the Wayamba University, while conducting your research at IRRI.

Your scholarship program is initially for a period of one (1) year effective on **2011 November 01** or on the date of your arrival at IRRI. An appropriate extension maybe considered depending on the progress of your academic work and availability of funding. Please note that the maximum duration of your program at IRRI is 3 years.

It is understood that your thesis research conducted at IRRI will be submitted to **WU** as a partial fulfillment for your PhD degree. Due recognition will be given to the fact that the thesis research was conducted at IRRI under the supervision of **Dr. Glenn Gregorio**. Also you are expected to write your thesis in English and to deposit a copy in the IRRI Library upon completion of your studies.

Funds for your scholarship will be provided to the International Rice Research Institute (IRRI) by the **2011 Global Rice Science Scholarship** under **Budget Code No. 7500-RC00000G4102090**. Under this program, you will receive the following benefits:

- roundtrip airticket by the most direct route to and from university where registered;
- pre- and post-departure allowance of US$75.00 (each way);
- funds to cover university fees;
- local group medical insurance up to a Maximum Benefit Limit (MBL) of Ps100,000.00 per disease/per year pro-rated based on your period of coverage, and accidental death insurance amounting to Ps500,000.00 (Philippines territory only and we encourage you to make arrangements for supplemental insurance protection to cover all travel, accident, medical, life, and other eventualities for which insurance would be desirable to have, inclusive of travel time to and from IRRI or any other location for this assignment.);
- a monthly stipend of US$700.00 for board, lodging, and incidental expenses during your stay at IRRI HQ;
- computer and network access at IRRI HQ;
- shipping allowance amounting to US$360.00;
- airport pick-up and send-off during travel to IRRI;
- AFSTRI membership fee;
- funds to cover book allowance amounting to US$300/year and;
- funds to cover attendance to professional development/leadership development courses while at IRRI; please note that attendance to such programs should be duly endorsed by your supervisors.

In addition, you will be provided office space, use of laboratory facilities and access to the Library and Documentation Center. Please note, however, that the program does not provide for family support whatsoever, and under no circumstances will IRRI assume any responsibility for your dependents in case they will join you. Moreover, you will not receive any other financial benefits from the scholarship whatsoever.

Mailing address DAPO 7777, 1301 Metro Manila, Philippines City and courier address Suite 1009, Security Bank Center, 6776 Ayala Ave., 1226 Makati City, Philippines
Tel +63 (2) 866-6133, 866-6123 Fax +63 (2) 891-1236 Los Baños Research Center Tel +63 (2) 580 5600, 845-0563, Fax +63 (2) 580-5699, 845-0606 Email: irri@cgiar.org Web: www.irri.org

Participants in IRRI's training programs are expected to return to their organizations immediately upon completion of their program and to work on rice and rice-based farming systems. They will not be considered for any additional training or reorientation program at IRRI during this period - unless cleared by their institute and with the IRRI Deputy Director General for Research (DDGR). IRRI will be unable to assist them in finding placement elsewhere.

Our travel agent, **BTI Philippines Marsman,** will prepare a visa cable to facilitate your 9E-2 visa application. Please send us your contact number, nearest Philippines Embassy where you will apply for your visa, and passport details (complete name, birth date/place, passport no., date/place of issue and validity) as soon as possible. Kindly note that you must obtain a "9E-2" visa to enter the Philippines and stay for the duration of your program.

By accepting a scholarship or training opportunity at IRRI, the scholar hereby agrees to the following:

Intellectual Property Rights

- *"results of collaborative research will be jointly published in the public interest as mutually agreed upon; and*
- that all outcomes of the **WU – IRRI** joint activities including all intellectual property rights (IPR) shall be jointly owned by all parties. As such, they shall remain in the public domain and shall be available to IRRI and institute partners and end users."

Entry and Exit of Biological and Non-biological Materials

If seeds or non seed biological materials (i.e. non seed plant parts, DNA samples, tissue/cell cultures of rice plants, etc.) need to be provided by your institution for work by you during your time at IRRI you must follow the following procedures:

- **you must not hand carry any material;**
- all materials must be shipped directly to IRRI and addressed to "Seed Health Unit, IRRI, College, Los Baños, Laguna, Philippines" with the necessary documents (i.e. Import Permit, Phytosanitary Certificate, Material Transfer Agreements, etc.);
- the Seed Health Unit, IRRI will assist you to obtain the necessary documents;
- you must directly coordinate with the Seed Health Unit, IRRI with copies to your concerned IRRI collaborating scientist and the Training Centre.

Safety

It is our understanding that you are fully aware of the need for safety both in the laboratory, field and controlled growth facilities, especially in relation to hazardous chemicals. By accepting this training opportunity at IRRI, you agree to strictly observe and comply with IRRI's safety protocols. Further, you agree to release and forever discharge IRRI and its employees from any and all claims, demands, actions and causes of action related to and/or arising from or incidental to your training participation.

If you find the terms and conditions of this award acceptable, please sign below and provide us a copy of the signed letter.

We look forward to your arrival at IRRI.

With best wishes.

Sincerely yours,

ACHIM DOBERMANN
Deputy Director General (Research)

I hereby agree:

DIKKUMBURAGE RADHIKA GIMHANI

ADVISER
WAYAMBA UNIVERSITY

Copy to: Dr. Bas Bouman; Dr. Bhagirath Chauhan; Ms. M. Aquino/Ms. R. San Gabriel; Mr. I. Cosico; IRRI Library; IRRI Front Desk.

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